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13. ABSTRACT (Maximum 200 words)

Progression of individual rates of age-dependent cognitive dysfunction and the potential for antioxidants and environmental enrichment to slow the rate of decline are being evaluated over a 3-year span in beagle dogs. The study started with dogs between the ages of 9 and 13 years. Dogs underwent baseline screening of cognitive function and general health evaluation including clinical pathology and physical examinations. Magnetic resonance image scans (MRIs) are being used to obtain *in vivo* measures of brain and cerebrovascular function. Each dog has been placed into one of four groups counterbalanced with respect to cognitive ability, sex, and age: 1) control, 2) enriched environment, 3) dietary enrichment, and 4) combined dietary and environmental enrichment. A broad spectrum of antioxidants is added for dietary enrichment. The environmental enrichment condition consists of additional cognitive experience and enriched sensory environment. Cognitive function, physical health, and brain MRIs are being monitored yearly to establish ongoing effects of the treatment. We now demonstrate significant improvements in learning and memory, particularly in animals receiving the combined treatment. In addition, we provide the first evidence that cognitive function can be maintained in treated animals, and hippocampal atrophy, measured by MRI volumetric analysis, has been slowed in animals receiving either or both of the interventions. We have now begun the neurobiological studies proposed for the study and are currently measuring the extent of β-amyloid deposition and several endpoint markers of oxidative damage. Neuroanatomical endpoint measures, once collected, will be correlated with cognitive function and MRI measures of brain atrophy and cerebrovascular function to establish the effectiveness of the treatments on delaying or preventing the development of age-dependent neuropathologies.

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I. INTRODUCTION

The purpose of the current research project was to determine the effects of both dietary antioxidants and behavioral enrichment on age-dependent cognitive decline and associated neuropathology in a 4-year longitudinal design using beagle dogs. Dogs underwent baseline screening of cognitive function and a general health evaluation including clinical pathology and physical examinations in the first year of the study. Magnetic resonance image (MRI) scans were used to obtain *in vivo* measures of brain and cerebrovascular function at four time points. For the cognitive testing portion of the study, a total of 24 aged animals from the Lovelace Respiratory Research Institute (LRRI), 7 young animals from LRRI, 24 aged animals from Hill's Pet Nutrition, Inc. (Hill's), and 8 young animals from Hill's were in the study. Aged dogs were placed in one of four treatment groups, which were counterbalanced with respect to baseline cognitive ability, sex, and age: (1) no behavioral enrichment/control diet group – C/C, (2) behavioral enrichment/control diet – E/C, (3) no behavioral enrichment/antioxidant diet – C/E, and (4) combined behavioral enrichment and antioxidant diet – E/A. For young animals, the housing conditions prevented assigning animals into a behavioral enrichment/control group. Thus, all young animals were behaviorally enriched but half received the antioxidant diet, and the other half received the control diet.

A broad spectrum of antioxidants and two mitochondrial co-factors were added to formulate an antioxidant-enriched geriatric canine diet. The behavioral enrichment condition consisted of additional cognitive experience, enriched sensory environment, and physical exercise. Cognitive function, physical health, and brain MRI scans were monitored annually to establish ongoing effects of the treatment. Aged animals from the LRRI colony (n = 24) were euthanized according to the study timeline, and anatomical and biochemical studies completed to date will be described. These anatomical studies will be used to establish the effectiveness of the treatments on delaying or preventing the development of age-dependent neuropathologies. Due to the lack of availability of the young animals for neurobiological studies, an additional five young beagle dogs from LRRI were included in the terminal phase of the study for comparisons with aged animals as described in the appropriate sections below.

II. BODY OF THE REPORT

In Year 5 we proposed to have completed the analysis of cognitive data, and we are continuing to analyze MRI data. A substantial portion of the anatomical/biochemical studies

initially proposed have been completed or are well underway. Additional studies have also been started using new markers as the field of study had developed considerably over the duration of the cognitive portion of the study.

A. Study Status

A total of 24 dogs from LRRI ranging in age from 9.3 to 13.8 years were placed into the study in October of 1998 and were supported by the current grant. Surviving animals were euthanized for anatomical and biochemical studies in May of 2002. A second group of 24 beagles ranging in age from 9.5 to 12.9 years from Hill's were added to the study in April of 1999 and were supported by Hill's. Only cognitive data and MRI data are available from the Hill's animals. At the time of this report, 13 Hill's animals were still alive. A set of young beagle dogs were also incorporated into the cognitive portion of the study supported on a National Institute on Aging (NIA) grant (AG12694) that allowed us to obtain comparative data from animals fed the control or antioxidant diet. Last, during the terminal phase of the experiments we included an additional five young beagle dogs from LRRI to use for comparative neurobiological studies as the young animals from the cognitive portion of the studies were in a survival study. These five young animals have been included in studies of mitochondrial function and other neurobiological studies. The list of animals included in the final cognitive, MRI, and in the neurobiological studies is provided in Table 1.

B. Summary of Cognitive Testing Results

The results of the cognitive testing procedures have been described in previous progress reports but will be summarized briefly here. In the first year of the study, animals were given a series of cognitive tests to evaluate learning, frontal lobe function, and memory. Based on scores from these tests, animals were placed into one of the four treatment groups such that each group contained both good and poor performers. Cognitive tasks proposed initially for the study were completed. In addition, new tests were subsequently developed and performed for age dependencee.

***Antioxidant diet effects on cognition*:** Animals placed in the behavioral enrichment condition were subsequently tested 6 days/week for the duration of the study. Thus, additional cognitive data were available from these animals but only allowed us to compare dogs fed the antioxidant diet to those fed the control diet. No conclusions regarding the effect of behavioral enrichment could be evaluated until the annual evaluation time point when all animals were tested. Within 2 weeks of beginning the antioxidant diet, rapid improvements in spatial attention were observed using a landmark discrimination task [1].

Table 1. List of animals included in the final cognitive, MRI, and neurobiological studies.

Dog	Date of End of Study	Intervention Start Date	Birthdate	Age at Start of Study (yr)	Current Age (yr)	Time on Intervention (yr)	Group		Source	Comments	Cognitive Data	MRI Data	Neuro-biological Data
							Diet	Environment					
1532S	05/15/02	08/06/99	02/09/89	10.49	13.27	2.78	Aox	Control	LRRI	Completed Study	Yes	Yes	Yes
1581S	05/16/02	09/13/99	05/15/91	8.34	11.01	2.67	Aox	Control	LRRI	Completed Study	Yes	Yes	Yes
1523B	05/15/02	09/13/99	11/26/89	9.80	12.47	2.67	Aox	Control	LRRI	Completed Study	Yes	Yes	Yes
1508A	05/15/02	08/06/99	02/12/88	11.49	14.26	2.78	Aox	Control	LRRI	Completed Study	Yes	Yes	Yes
1509U	01/21/02	08/06/99	03/03/88	11.43	13.90	2.46	Aox	Control	LRRI	Completed Study	Yes	Yes	Yes
1491B	05/14/02	09/13/99	05/13/87	12.35	15.01	2.67	Aox	Control	LRRI	Completed Study	Yes	Yes	Yes
1541B	05/16/02	09/03/99	05/25/89	10.28	12.98	2.70	Aox	Enriched	LRRI	Completed Study	Yes	Yes	Yes
1542T	05/14/02	08/06/99	06/03/89	10.18	12.95	2.77	Aox	Enriched	LRRI	Completed Study	Yes	Yes	Yes
1585A	05/15/02	09/13/99	08/29/91	8.05	10.72	2.67	Aox	Enriched	LRRI	Completed Study	Yes	Yes	Yes
1581T	05/14/02	09/13/99	05/15/91	8.34	11.01	2.67	Aox	Enriched	LRRI	Completed Study	Yes	Yes	Yes
1502S	05/14/02	08/06/99	08/16/87	11.98	14.75	2.77	Aox	Enriched	LRRI	Completed Study	Yes	Yes	Yes
1521B	05/15/02	09/13/99	10/06/88	10.94	13.61	2.67	Aox	Enriched	LRRI	Completed Study	Yes	Yes	Yes
1543S	05/17/02	07/18/99	06/04/89	10.13	12.96	2.83	Control	Control	LRRI	Completed Study	Yes	Yes	Yes
B2150	05/17/02	07/18/99	11/12/87	11.69	14.52	2.83	Control	Control	LRRI	Completed Study	Yes	Yes	Yes
1521S	05/17/02	08/15/99	10/06/88	10.86	13.62	2.76	Control	Control	LRRI	Completed Study	Yes	Yes	Yes
1494D	05/16/02	08/15/99	05/27/87	12.23	14.98	2.75	Control	Control	LRRI	Completed Study	Yes	Yes	Yes
1510A	05/17/02	09/13/99	03/22/88	11.48	14.16	2.68	Control	Control	LRRI	Completed Study	Yes	Yes	Yes
1508U	07/26/01	08/15/99	02/12/88	11.51	13.46	1.95	Control	Control	LRRI	Completed Study	Yes	Yes	Yes
1529S	05/16/02	07/18/99	01/23/89	10.49	13.32	2.83	Control	Enriched	LRRI	Completed Study	Yes	Yes	Yes
1523U	02/02/02	08/15/99	11/26/89	9.72	12.19	2.47	Control	Enriched	LRRI	Completed Study	Yes	Yes	Yes
1542S	05/16/02	07/18/99	06/03/89	10.13	12.96	2.83	Control	Enriched	LRRI	Completed Study	Yes	Yes	Yes
1506B	05/16/02	08/15/99	01/04/88	11.62	14.37	2.75	Control	Enriched	LRRI	Completed Study	Yes	Yes	Yes
1492B	11/24/99	08/15/99	05/23/87	12.24	12.52	0.28	Control	Enriched	LRRI	Off Study	Yes	Yes	Yes
1518D	05/17/02	07/18/99	09/18/88	10.84	13.67	2.83	Control	Enriched	LRRI	Completed Study	Yes	Yes	Yes
D056	10/15/02	01/31/00	12/05/88	11.16	13.87	2.71	Aox	Control	Hill's	Off Study	Yes	Yes	No
D048	10/15/02	01/31/00	09/15/88	11.38	14.09	2.71	Aox	Control	Hill's	Off Study	Yes	Yes	No
D064	10/15/02	01/31/00	08/15/89	10.47	13.18	2.71	Aox	Control	Hill's	Off Study	Yes	Yes	No
D067	10/15/02	01/27/00	10/01/90	9.33	12.05	2.72	Aox	Control	Hill's	Off Study	Yes	Yes	No
D081	10/15/02	02/07/00	02/23/90	9.96	12.65	2.69	Aox	Control	Hill's	Off Study	Yes	Yes	No
D082	10/15/02	01/31/00	09/18/91	8.38	11.08	2.71	Aox	Control	Hill's	Off Study	Yes	Yes	No
D058	10/03/00	02/07/00	09/20/88	11.39	12.04	0.65	Aox	Enriched	Hill's	Off Study	Yes	Yes	No
D060	10/15/02	01/31/00	09/20/89	10.37	13.08	2.71	Aox	Enriched	Hill's	Off Study	Yes	Yes	No
D054	10/15/02	01/27/00	05/15/90	9.71	12.43	2.72	Aox	Enriched	Hill's	Off Study	Yes	Yes	No
D055	10/15/02	01/27/00	10/16/88	11.29	14.01	2.72	Aox	Enriched	Hill's	Off Study	Yes	Yes	No

Table 1. List of animals included in the final cognitive, MRI, and neurobiological studies (Concluded).

Dog	Date of End of Study	Intervention Start Date	Birthdate	Age at Start of Study (yr)	Current Age (yr)	Time on Intervention (yr)	Group		Source	Comments	Cognitive Data	MRI Data	Neuro-biological Data
							Diet	Environment					
D065	10/15/02	01/27/00	06/10/89	10.64	13.36	2.72	Aox	Enriched	Hill's	Off Study	Yes	Yes	No
D075	10/15/02	01/27/00	02/08/90	9.97	12.69	2.72	Aox	Enriched	Hill's	Off Study	Yes	Yes	No
D051	10/15/02	01/15/00	08/15/89	10.42	13.18	2.75	Control	Control	Hill's	Off Study	Yes	Yes	No
D059	10/15/02	01/15/00	10/06/90	9.28	12.03	2.75	Control	Control	Hill's	Off Study	Yes	Yes	No
D062	10/20/01	01/15/00	10/01/90	9.30	11.06	1.76	Control	Control	Hill's	Off Study	Yes	Yes	No
D063	10/15/02	01/15/00	04/08/90	9.78	12.53	2.75	Control	Control	Hill's	Off Study	Yes	Yes	No
D066	10/15/02	11/20/99	05/28/90	9.49	12.39	2.90	Control	Control	Hill's	Off Study	Yes	Yes	No
D071	10/15/02	01/15/00	09/24/89	10.32	13.07	2.75	Control	Control	Hill's	Off Study	Yes	Yes	No
D052	10/15/02	02/07/00	07/08/88	11.59	14.28	2.69	Control	Enriched	Hill's	Off Study	Yes	Yes	No
D053	10/15/02	02/07/00	07/19/91	8.56	11.25	2.69	Control	Enriched	Hill's	Off Study	Yes	Yes	No
D080	10/15/02	02/07/00	08/04/89	10.52	13.21	2.69	Control	Enriched	Hill's	Off Study	Yes	Yes	No
D074	10/15/02	02/07/00	09/26/89	10.37	13.06	2.69	Control	Enriched	Hill's	Off Study	Yes	Yes	No
D073	10/15/02	02/07/00	09/21/89	10.39	13.07	2.69	Control	Enriched	Hill's	Off Study	Yes	Yes	No
D072	10/15/02	02/07/00	12/26/89	10.12	12.81	2.69	Control	Enriched	Hill's	Off Study	Yes	Yes	No
Apricot	10/15/03	10/17/99	03/12/98	1.60	5.60	4.00	Control	Enriched	LRRI	Longitudinal Study	Yes	Yes	No
Banana	10/15/03	10/17/99	08/10/97	2.19	6.18	4.00	Aox	Enriched	LRRI	Longitudinal Study	Yes	Yes	No
Cherry	10/15/03	10/17/99	10/08/97	2.02	6.02	4.00	Control	Enriched	LRRI	Longitudinal Study	Yes	Yes	No
Fig	10/15/03	10/17/99	03/12/98	1.60	5.60	4.00	Control	Enriched	LRRI	Longitudinal Study	Yes	Yes	No
Foster	10/15/03	10/17/99	07/16/95	4.26	8.25	4.00	Aox	Enriched	Hill's	Longitudinal Study	Yes	Yes	No
Genesis	10/15/03	07/28/00	10/03/97	2.82	6.04	3.22	Control	Enriched	Hill's	Longitudinal Study	Yes	Yes	No
Kiwi	10/15/03	10/17/99	12/10/96	2.85	6.85	4.00	Control	Enriched	LRRI	Longitudinal Study	Yes	Yes	No
Mango	10/15/03	10/17/99	12/10/96	2.85	6.85	4.00	Aox	Enriched	LRRI	Longitudinal Study	Yes	Yes	No
Phyllis	10/15/03	10/17/99	05/01/95	4.47	8.46	4.00	Control	Enriched	Hill's	Longitudinal Study	Yes	Yes	No
Reebok	10/15/03	07/28/00	12/08/97	2.64	5.85	3.22	Aox	Enriched	Hill's	Longitudinal Study	Yes	Yes	No
Sapphire	10/15/03	10/17/99	08/03/95	4.21	8.21	4.00	Aox	Enriched	Hill's	Longitudinal Study	Yes	Yes	No
Teamwolf	10/15/03	07/28/00	11/10/97	2.72	5.93	3.22	Aox	Enriched	Hill's	Longitudinal Study	Yes	Yes	No
Tuesday	10/15/03	07/28/00	10/15/97	2.79	6.00	3.22	Aox	Enriched	Hill's	Longitudinal Study	Yes	Yes	No
Wesley	10/15/03	10/17/99	05/31/95	4.38	8.38	4.00	Aox	Enriched	Hill's	Longitudinal Study	Yes	Yes	No
1633B	10/22/02	n/d	4/22/1998	0.00	4.50	0	Control	Control	LRRI	Neurobiology Study	No	No	Yes
1635B	10/22/02	n/d	5/30/1998	0.00	4.40	0	Control	Control	LRRI	Neurobiology Study	No	No	Yes
1637A	10/22/02	n/d	8/10/1998	0.00	4.20	0	Control	Control	LRRI	Neurobiology Study	No	No	Yes
1640T	10/22/02	n/d	9/18/1998	0.00	4.10	0	Control	Control	LRRI	Neurobiology Study	No	No	Yes
1648E	10/22/02	n/d	5/30/1999	0.00	3.40	0	Control	Control	LRRI	Neurobiology Study	No	No	Yes

Shaded cells are dogs being used in ongoing neurobiology studies.

After 6 months on treatment with the antioxidant diet, continued cognitive improvement was observed using a new test, called "oddity discrimination learning." This task is solved efficiently in young animals and is impaired in aged animals. Aged dogs receiving the antioxidant treatment showed significantly improved learning relative to untreated aged controls, particularly when the discrimination became more difficult [2]. Cognitive improving effects of the diet were maintained over the duration of the study and extended to other cognitive domains. Thus, after 1.5 years on treatment, dogs were given a more difficult conceptual learning task based on the size problem (see next paragraph), which was also shown to be age-dependent. Dogs fed the antioxidant diet performed significantly better than the dogs on the control diet [3].

Antioxidant diet, behavioral enrichment, and combination effects on cognition: To evaluate the effects of all four treatments, including the behavioral enrichment component, all dogs were tested on an annual basis. After 1 year on treatment, animals were tested for a size discrimination learning and reversal task, which allowed all four treatment groups to be compared. This task was intended to be more difficult than the baseline discrimination task to prevent "floor" effects. Logistic regression analysis revealed that both antioxidant-fortified food and behavioral enrichment led to improved performance in old dogs on both tasks, with the combined treatment group showing the greatest improvement [4]. After 2 years on the treatment, dogs were given a black/white discrimination learning and reversal task, which is sensitive to age. Discrimination learning was significantly improved by behavioral enrichment. Reversal learning was improved by both behavioral enrichment and dietary enrichment [manuscript in preparation].

Spatial memory was also evaluated each year of the study, beginning with baseline testing. We had initially proposed to also evaluate object recognition memory. However, the task proved too difficult for the older animals to learn and virtually all failed the task in the time allotted. This task was subsequently dropped from the protocol. After Year 1 and Year 2, spatial memory was re-tested and allowed the comparison of all four treatment groups. As reported in a previous progress report, spatial memory slowly improved over time in the antioxidant treated animals as well as animals receiving the behavioral enrichment protocol. The combination of the two treatments resulted in lower average error scores than either treatment alone [manuscript in preparation].

Summary: These results indicate that behavioral and dietary enrichment can work together in a synergistic fashion to attenuate age-dependent cognitive decline. Both interventions resulted in consistent and long-lasting improvements in cognitive function. The antioxidant supplement dose levels are well within ranges used in human studies and thus can be translated into pilot clinical trials to reduce age-associated and disease-associated cognitive impairments.

C. Health Examinations and Blood Biochemistry as a Function of Treatment

Medical evaluations of the dogs are completed through baseline and 3 years on study. These evaluations included physical examinations, blood samples for clinical chemistry and blood cell counts, and urinalysis. All of the LBERI/LRRI dogs were dead as of the last report. Four of the dogs died during the study period and 20 were sacrificed as scheduled. Four Hill's dogs died through 10/1/02 and 7 died during the past year. One dog each died in the control/control group, the enriched environment/control diet group, control environment/ antioxidant diet group; and 4 dogs died in the enriched environment/antioxidant group. The status of each group of dogs is given in Tables 2A–2D. No pattern of disease emerged, and no organ system appeared to have an increased attack rate.

The clinical chemistry and hematology data are given in Table 3 as means and 1 standard deviation for the dogs on the control diet and the antioxidant diet. The data were analyzed using repeated measures of variance, and those results are given in Table 4. Although there were significant differences for some parameters between diets, the mean values were all within normal ranges. There also were significant differences between sampling periods. Many of these changes reflect the change in values with increasing age. A few parameters had significant differences involving both time periods and diet. Again, the values remained within normal ranges and do not appear to represent any important trends. Thus, our interpretation of the clinical chemistry and hematology data are that there are age-related changes that occurred in these old dogs over the time of this study. The differences found between the control diet and the antioxidant diet were small and did not increase over time and thus may reflect some differences based on individual animals within these relatively small groups.

Clotting factors were measured at 2 and 2.5 years on study. The values are given in Table 5. No differences were found between the control and antioxidant diets. These values are the same as reported in last year's progress report.

Table 2A. Treatment Group: Control Environment/Control Diet

Dog Number	Colony of Origin	Alive	Sacrificed	Died	Age (yr)	Cause of Death
1494D	LRRI	no	5/16/02		14.9	
1508U	LRRI	no	–	7/26/01	12.4	Chronic heart failure
1510A	LRRI	no	5/17/02		13.2	
1521S	LRRI	no	5/17/02		12.6	
1543S	LRRI	no	5/17/02		11.9	
B2150	LRRI	no	5/17/02		13.5	
D051	Hill's	no	8/16/03		13.1	Liver cirrhosis
D059	Hill's	no		4/16/02	10.5	Hyperadrenocorticism
D062	Hill's	no		10/20/01	10.1	Chronic heart failure
D063	Hill's	10/1/03			12.5	
D066	Hill's	no		9/12/02	11.3	Discospondylosis
D071	Hill's	no		1/2/03	12.3	Disseminated tumor, mammary
	Totals	1	6	5		

Table 2B. Treatment Group: Enriched Environment/Control Diet

Dog Number	Colony of Origin	Alive	Sacrificed	Died	Age (yr)	Cause of Death
1492B	LRRI	no		11/24/99	12.5	Liver degeneration, pancreatitis and atrophy
1506B	LRRI	no	5/16/02		14.3	
1518D	LRRI	no	5/17/02		13.7	
1523U	LRRI	no		2/2/02	12.3	Chronic enteritis, nephritis
1529S	LRRI	no	5/16/02		13.3	
1542S	LRRI	no	5/16/02		12.9	
D052	Hill's	10/1/03			15.3	
D053	Hill's	10/1/03			13.3	
D072	Hill's	no		11/12/02	12.9	Discospondylosis, atrial wall fibrosis
D073	Hill's			4/17/02	12.6	Hemangiosarcoma, spleen
D074	Hill's	10/1/03			14.0	
D080	Hill's	10/1/03			14.1	
	Totals	4	4	4		

Table 2C. Treatment Group: Control Environment/Antioxidant Diet

Dog Number	Colony of Origin	Alive	Sacrificed	Died	Age (yr)	Cause of Death
1491B	LRRI	no	5/14/02		14.0	
1508A	LRRI	no	5/15/02		13.3	
1509U	LRRI	no		1/21/02	12.9	Abscess, left axilla
1523B	LRRI	no	5/15/02		11.5	
1532S	LRRI	no	5/15/02		12.3	
1581S	LRRI	no	5/16/02		10.0	
D048	Hill's	no		11/12/02	13.3	Valvular heart disease
D056	Hill's	10/1/03			13.8	
D064	Hill's	10/1/03			13.2	
D067	Hill's	10/1/03			12.0	
D081	Hill's	10/1/03			12.8	
D082	Hill's	10/1/03			11.0	
	Totals	5	5	2		

Table 2D. Treatment Group: Enriched Environment/Antioxidant Diet

Dog Number	Colony of Origin	Alive	Sacrificed	Died	Age (yr)	Cause of Death
1502S	LRRI	no	5/14/02		14.8	
1521B	LRRI	no	5/15/02		13.6	
1541B	LRRI	no	5/16/02		13.0	
1542T	LRRI	no	5/14/02		12.9	
1581T	LRRI	no	5/14/02		11.0	
1585A	LRRI	no	5/15/02		10.8	
D054	Hill's	no		6/10/03	13.1	Cholestatic disease
D055	Hill's	no		11/12/02	14.1	Valvular heart disease
D060	Hill's	no		4/17/03	13.6	Hemangiosarcoma, spleen
D065	Hill's	no		11/12/02	13.4	Atrial endocardial fibrosis
D070	Hill's	10/1/03			12.9	
D075	Hill's	no		4/23/03	13.1	Lymphosarcoma
	Totals	1	6	5		

Table 3. Means and one standard deviation for clinical chemistry and hematology values for dogs on the control or antioxidant diet for baseline and then every 6 months on the respective diets.

	Time in Years							
Parameters	Control-0	Antioxidant-0	Control-0.5	Antiox-0.5	Control-1.0	Antiox-1.0	Control-1.5	Antiox-1.5
AST (SGOT)	28.8 ± 8.9	26.5 ± 7.2	26.9 ± 5.4	27.8 ± 7.3	26.6 ± 5.8	25.9 ± 7.9	29 ± 5	31.4 ± 10.3
ALT (SGPT)	57 ± 84.6	45.2 ± 30.5	50.9 ± 47.8	41.3 ± 34.8	47.8 ± 40.3	51.2 ± 41.1	47.2 ± 28.4	50.8 ± 47
T. Bilirubin	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0	0.2 ± 0.1	0.1 ± 0	0.1 ± 0.1	0.1 ± 0	0.1 ± 0
Alk Phos	176.2 ± 115.8	144.5 ± 92.9	173.3 ± 100.8	171.3 ± 117.9	174.3 ± 133.7	185.7 ± 130.5	206.8 ± 183.3	173.9 ± 118.2
GGT	5.1 ± 5.1	5.3 ± 4.1	4.5 ± 4.3	5 ± 4.5	3 ± 2.6	5.5 ± 7.7	4.6 ± 8.8	4.8 ± 7.7
Total Protein	6 ± 0.5	6 ± 0.4	6.3 ± 0.4	6.1 ± 0.3	6.3 ± 0.5	6 ± 0.3	6.1 ± 0.5	6.1 ± 0.4
Albumin	3.1 ± 0.3	3.2 ± 0.3	3.3 ± 0.3	3.4 ± 0.2	3.3 ± 0.4	3.3 ± 0.2	3 ± 0.4	3.2 ± 0.3
Globulin	3 ± 0.5	2.8 ± 0.4	3 ± 0.5	2.7 ± 0.3	3 ± 0.4	2.7 ± 0.3	3.1 ± 0.5	2.9 ± 0.4
Cholesterol	230.1 ± 70.8	228.5 ± 61.2	203.1 ± 49.1	238.4 ± 63	218 ± 82.2	235 ± 49.1	226.1 ± 68.8	243.8 ± 51.3
BUN	11.8 ± 3.3	13.9 ± 4.4	11.5 ± 3.2	12.2 ± 2.8	12.5 ± 4.9	12.8 ± 4.3	11.8 ± 4.4	12.4 ± 3.3
Creatinine	0.9 ± 0.2	1 ± 0.2	0.7 ± 0.2	0.7 ± 0.2	0.7 ± 0.2	0.8 ± 0.2	0.7 ± 0.1	0.8 ± 0.1
Phosphorus	4.2 ± 0.7	4.2 ± 0.7	4.1 ± 1.2	3.8 ± 0.8	3.9 ± 0.6	4 ± 0.8	4.1 ± 0.8	4.1 ± 0.9
Calcium	9.5 ± 0.3	9.6 ± 0.5	9.7 ± 0.7	9.8 ± 0.5	9.9 ± 0.6	9.5 ± 0.5	9.6 ± 0.7	9.5 ± 0.5
Glucose	85.9 ± 9.2	87.2 ± 8.8	85.8 ± 9.1	86 ± 10.3	91.4 ± 8.8	90 ± 11.2	93 ± 11.3	94.1 ± 8.5
Amylase	669 ± 178.4	669 ± 178.8	689 ± 182.1	670.8 ± 144.9	709.7 ± 225.8	656.7 ± 174.6	721.5 ± 200.8	716.2 ± 210.1
Lipase	399.6 ± 188.4	314.7 ± 148	354 ± 153.4	276.7 ± 147.4	347.7 ± 133.3	258.5 ± 120.5	335.3 ± 154	268.8 ± 133.3
Sodium	143.9 ± 2.5	144.8 ± 2.4	145.3 ± 2.7	144 ± 2.4	146.1 ± 1.9	145.1 ± 2.4	144 ± 1.8	145.3 ± 1.9
Potassium	4.5 ± 0.3	4.3 ± 0.2	4.6 ± 0.3	4.4 ± 0.2	4.6 ± 0.3	4.3 ± 0.3	4.7 ± 0.3	4.6 ± 0.3
Chloride	110.4 ± 3.2	111.3 ± 4.1	109.3 ± 2.2	109.6 ± 3	109.1 ± 4	109.1 ± 3.6	110.4 ± 3.5	111.3 ± 3.7
CPK	172 ± 75.5	124.1 ± 45.4	143.3 ± 56.5	136.4 ± 51.5	142 ± 51.8	121.1 ± 51.6	150 ± 47.3	128.3 ± 57.2
Triglyceride	148.2 ± 107.4	125 ± 123.4	120.1 ± 64.3	90.8 ± 60.2	113.9 ± 88.7	88.2 ± 63.1	150.7 ± 99.7	132.9 ± 83.8
Osmolality- Calc	296.7 ± 5	299.5 ± 5.6	299.6 ± 5.2	297.2 ± 5.1	301.7 ± 4.4	299.8 ± 5.4	297.4 ± 4.2	300.3 ± 4.2
Corrected CA	10 ± 0.3	9.9 ± 0.5	9.8 ± 0.4	9.9 ± 0.6	9.7 ± 1.3	9.6 ± 0.4	10 ± 0.6	10 ± 0.4
Magnesium	.	.	1.7 ± 0.3	1.9 ± 0.3	1.8 ± 0.9	1.7 ± 0.2	1.5 ± 0.2	1.7 ± 0.2
WBC	8.2 ± 3.4	7.1 ± 2.2	8.3 ± 1.8	7.1 ± 1.9	8.3 ± 2.2	6.8 ± 1.9	10.2 ± 4.7	8 ± 1.8
RBC	7 ± 0.5	6.8 ± 0.8	6.9 ± 0.6	7 ± 0.8	8.3 ± 7	6.7 ± 0.6	6.6 ± 0.7	6.9 ± 0.6
HGB	16.1 ± 1.6	15.8 ± 1.9	15.8 ± 1.4	15.9 ± 1.2	18 ± 11.3	15.2 ± 1.3	15.1 ± 1.8	15.8 ± 1.2
PCV	51.5 ± 4.6	50 ± 5.2	47.8 ± 4	48.1 ± 4	46.2 ± 7.2	46 ± 3.6	45.6 ± 4.7	47.4 ± 3.6
MCV	73.3 ± 5.1	74.2 ± 3.1	69.3 ± 2.3	69.6 ± 3.1	67.5 ± 7.8	68.8 ± 3.3	68.6 ± 2.2	68.6 ± 3
MCH	23 ± 1.3	23.5 ± 1	22.9 ± 1.1	22.9 ± 1.3	179.2 ± 749.9	22.8 ± 1.3	22.8 ± 1.2	22.9 ± 1.5
MCHC	31.3 ± 1.1	31.8 ± 0.9	33.2 ± 0.7	33 ± 0.6	64.5 ± 149.9	33.3 ± 0.8	33.3 ± 1.3	33.3 ± 1
Neuts	5382.6 ± 3030	4484.5 ± 1895.3	6009.3 ± 1394.4	5331.3 ± 1495.9	6024.4 ± 2021.6	5091.1 ± 1438.9	8039.1 ± 4583.9	6039.9 ± 1748.3
Lymphs	2022.3 ± 714.2	1800.4 ± 453.8	1582.2 ± 567.8	1192.6 ± 398.1	1263.4 ± 505.8	1176.4 ± 620.9	1397.5 ± 513	1252.7 ± 382.2
Monos	319 ± 196.4	264 ± 96.7	606.7 ± 215.1	525.9 ± 224.5	474.4 ± 165.6	444.8 ± 191.9	625.1 ± 320	582.8 ± 211.5
Eosinophils	463.6 ± 243.3	501.1 ± 210.7	228.3 ± 304.1	225 ± 184.7	389.6 ± 650.1	164.4 ± 85.4	240.4 ± 164.8	547.8 ± 641
Basophils	0 ± 0	0 ± 0	90.7 ± 16.7	53.6 ± 39.3	41.2 ± 45.2	16.4 ± 24.9	31.4 ± 43	71.3 ± 38.4
T3 (RIA)	93.6 ± 22	94 ± 20.4	96 ± 25	102.5 ± 20.7	86 ± 21.3	104.8 ± 21.2	86.2 ± 25.1	98 ± 20.2
T4 (RIA)	1.5 ± 0.5	1.7 ± 0.4	1.3 ± 0.4	1.2 ± 0.4	1.5 ± 0.5	1.6 ± 0.6	1.2 ± 0.4	1.5 ± 0.5
Free T4 (RIA)	1.2 ± 0.4	1.4 ± 0.3	1.2 ± 0.3	1.1 ± 0.3	1.2 ± 0.3	1.4 ± 0.4	0.6 ± 0.3	0.7 ± 0.3
T3AA	1 ± 0.2	1 ± 0.3	1.2 ± 0.7	1.1 ± 0.4	1.1 ± 0.5	1.2 ± 0.8	0.8 ± 0.3	1.2 ± 1.1
T4AA	0.9 ± 0.1	0.8 ± 0.1	0.9 ± 0.2	0.8 ± 0.2	0.8 ± 0.2	0.7 ± 0.2	0.9 ± 0.1	0.9 ± 0.3
Free T3	3 ± 0.6	3.1 ± 0.8	2.9 ± 0.8	2.9 ± 0.8	4 ± 1.5	3.5 ± 1.4	3.7 ± 1.2	4.7 ± 1.3
TSH	0.3 ± 0.5	0.3 ± 0.7	0.5 ± 0.8	0.5 ± 0.7	0.6 ± 0.8	0.6 ± 1.1	0.5 ± 0.7	0.5 ± 0.8
Quant. Platelet	316.9 ± 85.8	302.9 ± 75.7	282.9 ± 100.7	280 ± 87.1	339.9 ± 88.6	259.1 ± 80.9	332.3 ± 104.6	264.3 ± 90.4

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Table 3. Means and one standard deviation for clinical chemistry and hematology values for dogs on the control or antioxidant diet for baseline and then every 6 months on the respective diets (Concluded).

	Time in Years					
Parameters	Control-2.0	Antiox-2.0	Control-2.5	Antiox-2.5	Control-3.0	Antiox-3.0
AST (SGOT)	37.5 ± 18.2	32 ± 8.4	28.5 ± 5.6	31.8 ± 12	24.1 ± 7.6	23.4 ± 7.5
ALT (SGPT)	74.8 ± 82	35.6 ± 10.1	46.6 ± 32.2	50 ± 37.5	42.4 ± 20.9	53.9 ± 52.6
T. Bilirubin	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0
Alk Phos	145.6 ± 148.2	159.3 ± 100.1	2859 ± 10712.8	184.2 ± 88.2	309.2 ± 638.9	158.8 ± 83.9
GGT	5.4 ± 5.8	1.7 ± 1.4	9.2 ± 12.5	8.1 ± 8.4	4.4 ± 2.3	5.5 ± 5.3
Total Protein	6.4 ± 0.4	6 ± 0.4	6 ± 0.7	5.9 ± 0.4	5.7 ± 0.6	5.2 ± 0.6
Albumin	3 ± 0.3	3.1 ± 0.3	2.9 ± 0.5	3 ± 0.3	2.8 ± 0.4	2.7 ± 0.3
Globulin	3.4 ± 0.5	2.9 ± 0.3	3.1 ± 0.7	2.9 ± 0.4	2.9 ± 0.6	2.5 ± 0.3
Cholesterol	226.4 ± 44.7	256.7 ± 57.6	212.9 ± 58.5	229.5 ± 52.3	183.4 ± 40.3	193.8 ± 31.4
BUN	11.6 ± 2.2	11.9 ± 3.2	14.2 ± 5.6	13.8 ± 4.7	14.9 ± 6	14.8 ± 5.2
Creatinine	0.7 ± 0.1	0.8 ± 0.2	0.7 ± 0.2	0.7 ± 0.2	0.9 ± 0.3	0.8 ± 0.2
Phosphorus	3.7 ± 0.8	4.3 ± 0.6	4.7 ± 0.7	4.5 ± 1	4.6 ± 0.7	4.9 ± 1.1
Calcium	9.7 ± 0.5	10 ± 0.6	10.1 ± 1.3	10.2 ± 0.7	9.9 ± 0.4	9.6 ± 0.5
Glucose	88.9 ± 13.1	97.2 ± 13.1	87.2 ± 11.2	88.8 ± 11.3	99.2 ± 22.6	98.7 ± 22.7
Amylase	758.9 ± 160.7	795.2 ± 222.3	736.4 ± 280.5	737.8 ± 168.3	703.8 ± 235.8	726.9 ± 234.1
Lipase	282.2 ± 137.3	283.8 ± 170	515.1 ± 796.2	308.9 ± 185.6	651 ± 1268.6	305 ± 214
Sodium	146.3 ± 1.3	146.1 ± 1.6	148.4 ± 4.2	149.9 ± 4.5	149.8 ± 4	144.9 ± 2.5
Potassium	4.9 ± 0.3	4.6 ± 0.3	4.9 ± 0.5	4.4 ± 0.4	4.7 ± 0.5	4.3 ± 0.3
Chloride	112.5 ± 3.6	113.5 ± 3.4	113.5 ± 4.2	115 ± 5.5	115.1 ± 5.2	112.9 ± 3.7
CPK	176.2 ± 29.4	165.1 ± 96.3	154.9 ± 49.9	135.7 ± 54.4	153.9 ± 99.6	116.2 ± 22.7
Triglyceride	130.6 ± 47.7	107.4 ± 76.8	124.1 ± 147.8	92.5 ± 58.3	111.7 ± 55.2	99.5 ± 65.7
Osmolality- Calc	301.6 ± 2.5	301.7 ± 3.7	301.8 ± 4.7	304.4 ± 5.3	306.4 ± 12.7	273.7 ± 73.1
Corrected CA	10.3 ± 0.3	10.3 ± 0.3	10.6 ± 0.9	10.6 ± 0.7	10.7 ± 0.5	10.4 ± 0.3
Magnesium	1.7 ± 0.3	1.6 ± 0.1	1.7 ± 0.2	1.8 ± 0.2	1.6 ± 0.1	1.7 ± 0.1
WBC	8.5 ± 2.1	8.2 ± 1.9	8.9 ± 2.3	7.8 ± 2.4	6.6 ± 4.7	6 ± 4
RBC	6.6 ± 0.8	6.8 ± 1.1	5.7 ± 0.6	6.2 ± 1	5 ± 0.9	5.4 ± 0.9
HGB	14.8 ± 2	15.3 ± 1.8	13.5 ± 1.9	14.2 ± 2.2	11.4 ± 2.2	12.3 ± 1.9
PCV	44.3 ± 5.4	44.9 ± 5.5	40.1 ± 5.4	41.9 ± 6.4	35.6 ± 7.9	37.9 ± 6.9
MCV	67.7 ± 2.3	66.7 ± 4.1	69.9 ± 3.4	68.2 ± 4.7	70.9 ± 4.7	69.8 ± 4.6
MCH	22.6 ± 1	22.8 ± 1.9	23.6 ± 1	23.1 ± 1.6	22.9 ± 0.8	22.8 ± 1.6
MCHC	33.6 ± 0.7	34.3 ± 0.9	33.8 ± 1.1	33.8 ± 1	32.4 ± 1.3	32.7 ± 1.3
Neuts	6033.2 ± 1504.1	6322.8 ± 1378.5	6837.6 ± 2098.3	5830.3 ± 1842.6	4637.9 ± 3529.5	4279.1 ± 2999.8
Lymphs	1517.9 ± 542.2	965.3 ± 671.8	1178.2 ± 378.2	1113.1 ± 331.1	1232.5 ± 707.8	974.4 ± 598.1
Monos	725.5 ± 265.5	729.7 ± 291.1	648.6 ± 268.2	596.3 ± 285.1	351.4 ± 168.1	353.5 ± 258.4
Eosinophils	218.3 ± 243	639.5 ± 508.4	336.6 ± 255.8	287.8 ± 214.8	399.6 ± 478.1	564.5 ± 551.9
Basophils	90.3 ± 12.7	0 ± 0	89.8 ± 22.1	84.1 ± 28.1	0 ± 0	97 ± 0
T3 (RIA)	89.8 ± 21.6	108.3 ± 29	82.5 ± 14.6	94 ± 18.2	73.4 ± 19.9	85.5 ± 21.3
T4 (RIA)	0.9 ± 0.3	1.2 ± 0.8	1.2 ± 0.6	1.2 ± 0.5	1 ± 0.4	1.1 ± 0.7
Free T4 (RIA)	0.4 ± 0.2	0.5 ± 0.3	0.5 ± 0.2	0.6 ± 0.2	0.5 ± 0.3	0.6 ± 0.4
T3AA	0.8 ± 0.2	1.1 ± 0.4	1 ± 0.2	1 ± 0.3	0.9 ± 0.1	1 ± 0.3
T4AA	1.1 ± 0.3	1.1 ± 0.4	1.3 ± 0.3	1.3 ± 0.3	1.3 ± 0.1	1.2 ± 0.2
Free T3	3.4 ± 1	3.6 ± 1	3.3 ± 0.5	4.1 ± 0.6	2.6 ± 0.3	2.4 ± 0.7
TSH	0.7 ± 1.3	0.4 ± 0.2	0.6 ± 1.1	0.6 ± 0.9	0.3 ± 0.2	0.3 ± 0.3
Quant. Platelet	370 ± 128.8	370.5 ± 124.9	336.8 ± 101.8	308.8 ± 95.8	385.9 ± 160.3	317.8 ± 108.5

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Table 4. Statistical differences between control diet and antioxidant diet, among the various time periods or among diet and tissue period.

	Diet	Time Period	Time Period and Diet
AST (SGOT)		X	
ALT (SGPT)			
T. Bilirubin		X	
Alk Phos			
GGT		X	
Total Protein	X	X	
Albumin		X	
Globulin	X	X	
Cholesterol	X	X	
BUN		X	
Creatinine		X	
Phosphorus		X	
Calcium		X	
Glucose		X	
Amylase			
Lipase	X		
Sodium	X	X	X
Potassium	X	X	
Chloride		X	
CPK	X		
Triglyceride	X		
Osmolality- Calc	X	X	X
Corrected CA			
Magnesium		X	
WBC	X	X	
RBC		X	
HGB		X	
PCV		X	
MCV		X	
MCH			
MCHC			
Neuts	X	X	
Lymphs	X	X	
Monos		X	
Eosinophils			
Basophils		X	X
T3 (RIA)	X	X	
T4 (RIA)		X	
Free T4 (RIA)		X	
T3AA			
T4AA		X	
Free T3		X	X
TSH			
Quant. Platelet	X	X	

Table 5. Clotting factor values for dogs on the control or antioxidant diet for 2 to 2.5 years. Values are means and one standard deviation.

	Time in Years			
Parameters	Control-2.0	Antiox-2.0	Control-2.5	Antiox-2.5
PT	8.3 ± 1.9	7.6 ± 1.5	6.9 ± 1	6.4 ± 0.4
aPTT	11.8 ± 1.2	12.5 ± 0.7	13.5 ± 0.8	12.8 ± 0.4
FIB	310.7 ± 84	272.8 ± 100.8	349.1 ± 89.8	315.9 ± 88.9

PT = prothrombin time; aPTT = activated partial thromboplastin time; FIB = fibrogen

Vitamin E levels remain high in dogs provided with the antioxidant diet. As previously reported, the animals receiving the diet rich in antioxidants continue to maintain significantly high vitamin E levels relative to control diet animals [$(F(3,41) = 12.91, p < 0.0001)$, see Figure 1]. The analysis of the final vitamin E samples is pending at this time.

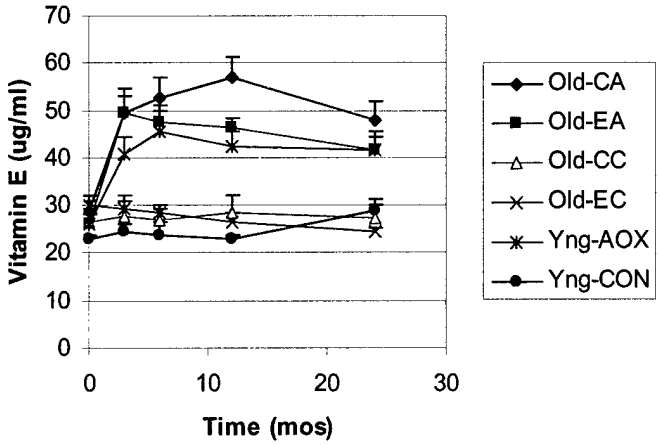

Figure 1. Serum vitamin E levels over time for old and young dogs on vitamin E-enriched diet or control diet.

D. Dynamic Contrast Enhanced Magnetic Resonance Imaging—Changes in Brain Volume as a Function of Age and Treatment

Each year of the study we have obtained *in vivo* measures of anatomical parameters and cerebrovascular function to detect longitudinal age changes and response to treatment. These experiments were included in the initial proposal, and additional financial support from the NIA was obtained to include more animals (Hill's) and for the extended study design. The rationale for including MRI scans was that a prior study showed age-dependent changes in volumetric measures [5] and that this technique could provide a noninvasive method for monitoring aging and treatment effects. In total, four scans of each dog were completed. Within 1 month prior to euthanasia, animals were given their last MRI scan. These final scans were used along with the prior scans to compare animals and treatments across all years of the study. The imaging study has generated an extensive data set and is currently being analyzed with assistance from a biostatistician. We will provide a summary of the outcome of the current analyses in this section.

Methods: We have described detailed methods in previous progress reports. The MRI experiments were performed on a GE Signa 1.5 T scanner with a linear head coil as described previously [5]. Each animal was anesthetized by inhalation of isoflurane (1.5–2%) throughout the experimental procedure. A set of three-dimensional images across the whole brain were acquired using a spoiled gradient refocus pulse sequence (SPGR) to obtain the detailed anatomic images.

Results: Two regions of interest were outlined in consecutive coronal slices from each animal for each time point. These two regions included the lateral ventricles and the hippocampus. Volumes for each region were subsequently normalized with respect to total intracranial volume. A repeated-measures ANOVA was used to determine the effect of time (within subject measures) and treatment (between subjects). Over the 4 years of scans, overall the ventricles in all dogs showed a general increase in volume ($F(3,87) = 26.77$, $p < 0.0001$). However, during the first 2 years of the study, ventricular size was relatively stable. In Year 3 of the study, ventricle volume increased dramatically and increased further in the last year of the study (Figure 2A). The majority of this effect was due to aged animals in the control/control or behavioral enrichment/control group showing larger volumes than animals receiving the antioxidant diet ($F(1,29) = 5.46$, $p < 0.027$) (Figure 2B).

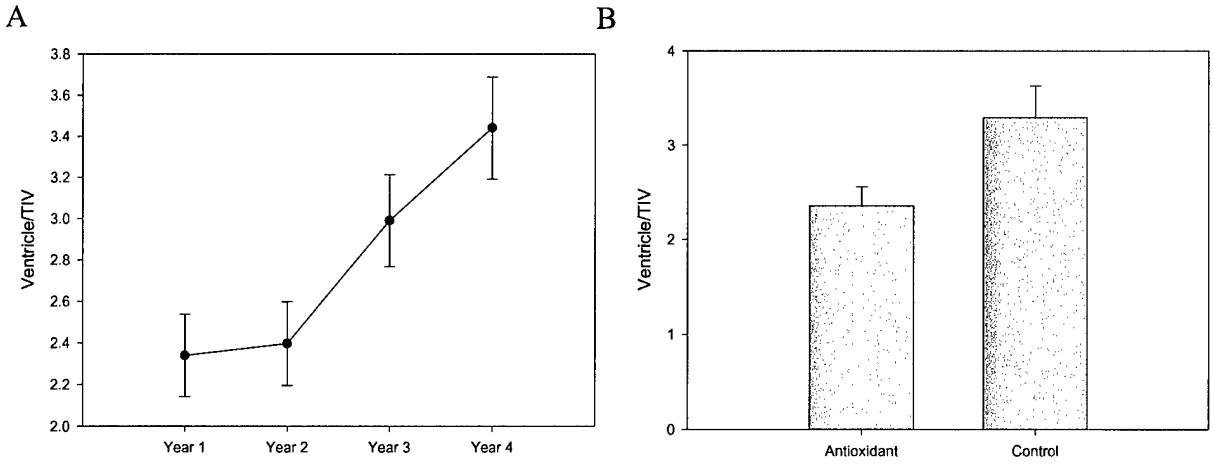

Figure 2. Ventricular volume measured by dynamic contrast enhanced magnetic resonance imaging increases with age and is reduced in antioxidant-fed dogs. A. The mean ventricular volume for all animals in the study remained stable in Years 1 and 2 of the study but rapidly increased in years 3 and 4. B. Most of this effect was due to control animals as a comparison of the extent of ventricular widening in Year 4 of the study between aged animals fed the antioxidant diet compared to the control diet animals shows smaller ventricular volumes in the treated group. Ventricular volume is thought to reflect global cortical atrophy. TIV = total intracranial volume, error bars = standard errors of the mean.

An opposite effect was observed for the hippocampus (Figure 3). Over time, the hippocampal volume increased slightly from Year 1 to Year 2. However, in Years 3 and 4, the volume of the hippocampus declined ($F(3,87) = 20.96$, $p < 0.0001$) (Figure 3A). Most of the hippocampal volume reduction was attributable to the animals receiving the control diet whereas antioxidant-treated animals maintained higher hippocampal volumes ($F(1,29) = 6.26$ $p < 0.018$) (Figure 3B). Representative images from one animal in each treatment condition are provided to show differences in ventricular and hippocampal volume (Figure 4).

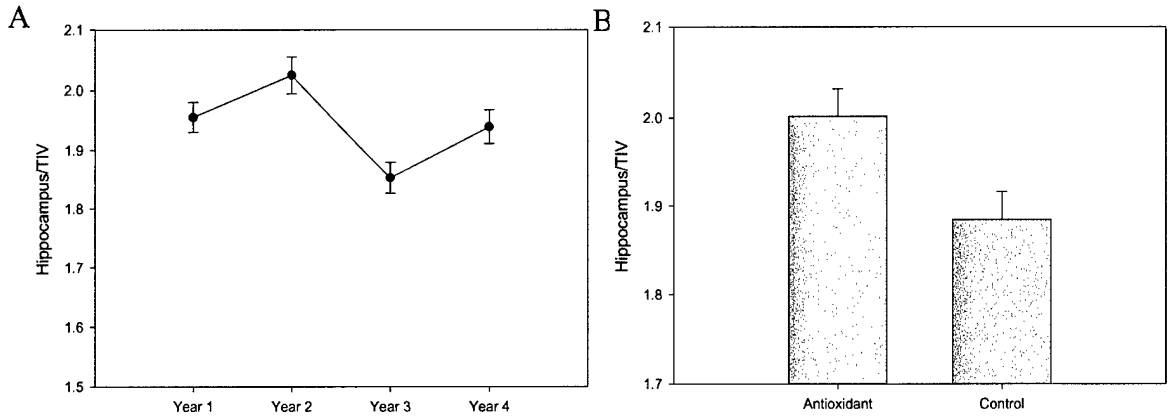

Figure 3. Hippocampal volume measured by dynamic contrast enhanced magnetic resonance imaging decreases with age and is maintained in antioxidant-fed dogs. A. The mean hippocampal volume for all animals is plotted as a function of year of the study. As with changes in ventricular volume, hippocampal volumes remained relatively stable in the first two years of the study but decreased in Years 3 and 4. B. The majority of this effect was due to control dogs because hippocampal volume was higher in antioxidant-fed animals in Year 4 of the study. TIV = total intracranial volume, error bars = standard errors of the mean.

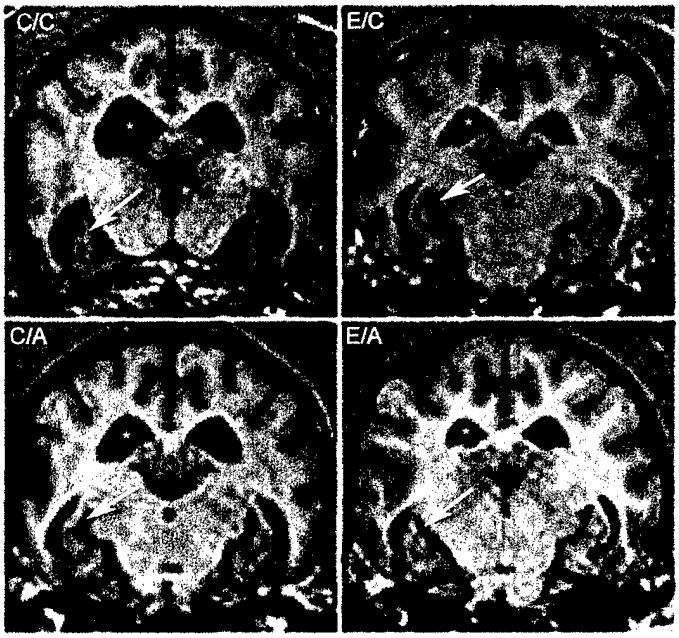

Figure 4. Representative images from an animal in each treatment group illustrating ventricular widening and hippocampal atrophy in untreated animals relative to dogs receiving treatment. Arrows point to hippocampus and asterisk indicates lateral ventricles. C/C = control enrichment/control diet, E/C = cognitive enrichment/control diet, C/A = control enrichment/antioxidant diet, E/A = behavioral enrichment/antioxidant diet.

Other brain regions are currently under analysis as are the cerebrovascular function measures. We anticipate these results to be fully analyzed over the next 6 months.

Summary: Longitudinal age-dependent increases in ventricular volume may indicate a general atrophy of cortical structures over time. These age effects were reduced by providing animals with a diet rich in antioxidants but not by providing behavioral enrichment. Treating animals with a diet rich in antioxidants could also slow age-dependent declines in hippocampal volume. Thus, on gross measures of brain anatomical volumes, both longitudinal age effects and treatment effects were observed. Also, most of the more dramatic age changes occurred after Year 2 of the study, highlighting the usefulness of longitudinal (as opposed to cross-sectional) imaging studies. This is one of the first studies to show treatment effects on the aging brain in a higher mammalian model. Noninvasive imaging techniques such as the one described here can be directly translated into human clinical trials and will be useful for monitoring antioxidant treatment effects.

E. The Production of Reactive Oxygen Species by Mitochondria Is Higher in Aged Animals and Is Reduced by an Antioxidant and Mitochondrial Co-Factor Enriched Diet

Oxidative damage is caused by free radicals that are generated primarily by mitochondria [6]. Two components of this enriched diet were lipoic acid and carnitine, which are both mitochondrial co-factors, and we hypothesized that these two components would improve mitochondrial function. L-carnitine is a precursor to acetyl-l-carnitine and is involved in mitochondrial lipid metabolism and maintaining efficient brain function [7]. Alpha-lipoic acid is a co-factor for the mitochondrial respiratory chain enzymes, pyruvate and alpha-ketoglutarate dehydrogenases, as well as an antioxidant capable of redox recycling of other antioxidants and raising intracellular glutathione levels [8]. We hypothesized that animals treated with an antioxidant diet for an extended period of time would show less evidence of mitochondrial dysfunction measured as a reduction in generation of reactive oxygen species (ROS). These experiments were not proposed in the initial design, but through collaboration with an expert on mitochondrial function (Dr. Patrick Sullivan, University of Kentucky), we were able to conduct a unique experiment to evaluate the effects of the dietary and behavioral enrichment treatments directly on isolated mitochondria.

Methods: Mitochondrial samples were obtained by biopsy and measures of ROS production were completed immediately after isolating mitochondria. Prior to euthanasia, the

surviving 20/24 aged dogs and all five young dogs underwent a cortical biopsy procedure. Animals were sedated with 0.2 mg/kg acepromazine given subcutaneously 20 min before induction of general anesthesia. General anesthesia was induced with 5% isoflurane by inhalation. An endotracheal tube was then placed in each dog, and the surgical level of anesthesia maintained with 2–3% isoflurane in oxygen. An incision was made just to the right of the sagittal crest over the parietal and frontal bones to the external frontal crest and then down on each end to form a flap. The muscles were then dissected away from the bones covering right parietal cortex region. A bone saw was used to cut a small 3 × 3 cm piece of the parietal bone, and this was removed to expose the underlying meninges and brain. Cautery was used to cut the meninges and to provide hemostasis in the sample site. A sterile metal "scoop" was used to dissect out a 2 × 1 × 1 cm block of parietal cortex tissue. Dogs were maintained under anesthesia until the final euthanasia procedure.

Mitochondrial Isolation. Isolated brain mitochondria were prepared as previously described [9, 10]. Mitochondrial ROS production was measured using the indicator dichlorodihydrofluorescein diacetate (H_2DCFDA, molecular probes) as previously described [10]. Oxidative modification of isolated mitochondrial proteins was measured by assessment of protein carbonyl content. The carbonyls were derivatized with dinitrophenylhydrazine (DNPH) followed by dot blot using an ECL Western blotting kit according to the manufacturer's protocol (Amersham Pharmacia Biotech, Uppsala, Sweden), the films scanned, and the densitometric measurements quantified [11].

Results: ROS production varied both as a function of age and of treatment, as shown in Figures 5–7. A repeated-measures ANOVA (time – 5, 10, 15 minutes) was used to compare young dogs with untreated old animals and to test for treatment effects. There was a significant main effect of time on ROS

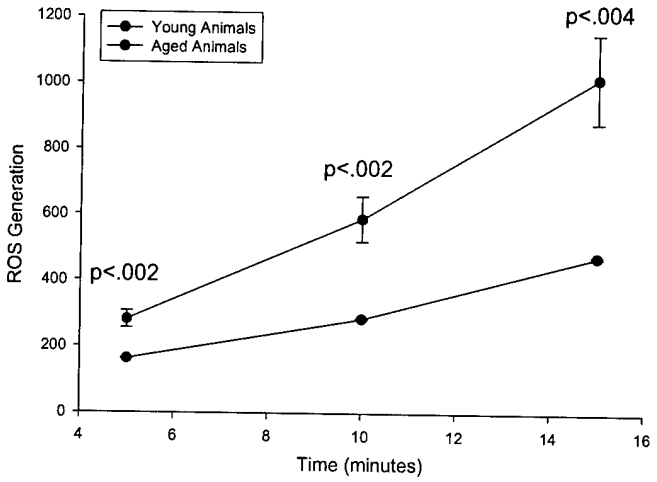

Figure 5. Age-dependent increase in reactive oxygen species production by canine mitochondria. The extent of ROS generation is plotted as a function of time for aged and young beagles separately. At all time points, the mitochondria of aged dogs produced more ROS than young dogs. Bars represent standard errors of the mean.

measures ($F(2,16) = 90.92$, $p < 0.0001$), indicating a rise in ROS production with increasing time. Young animals showed significantly lower levels of ROS production than untreated aged animals ($F(1,8) = 17.65$, $p < 0.003$) and a significant age-by-time interaction ($F(2,16) = 14.91$, $p < 0.0001$). These effects reflect a more rapid accumulation of ROS production within the aged group of animals compared with the young animals over time (Figure 5).

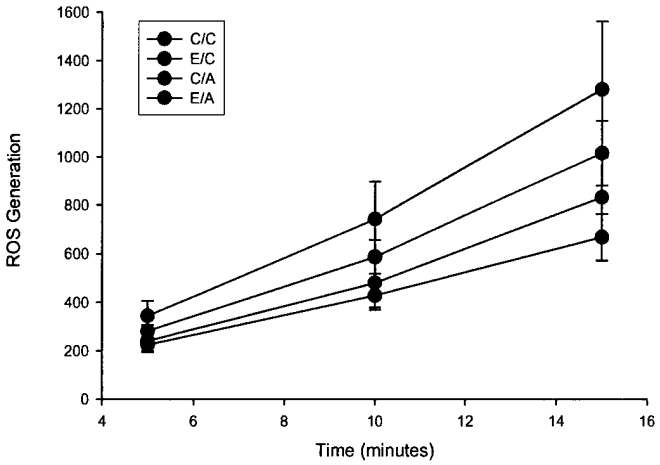

Figure 6. Mitochondrial ROS production varies as a function of treatment in aged dogs. The extent of ROS produced over time is plotted separately for each treatment condition. Note that untreated aged controls produced more ROS than dogs receiving the antioxidant diet. Bars indicate standard errors of the mean. C/C = control enrichment/control diet, E/C = cognitive enrichment/control diet, C/A = control enrichment/ antioxidant diet, E/A = behavioral enrichment/antioxidant diet.

A similar repeated-measures ANOVA was used to analyze treatment effects within the aged group of dogs. As with young animals, a significant rise in ROS production was observed across time ($F(2,28) = 99.67$, $p < 0.0001$). A significant main effect of treatment group was not observed. However, there was a significant treatment group-by-time interaction ($F(6,28) = 2.42$, $p < 0.051$), indicating that the animals receiving only the behavioral enrichment showed the highest ROS generation overall but particularly at the long time delays (Figure 6). However, the two antioxidant diet conditions showed lower rates of ROS production. Thus, a second analysis was used to compare animals receiving the diet rich in antioxidant to those receiving the control diet. Both groups showed increasing ROS production over time ($F(2,32) = 99.56$, $p < 0.001$). There was also a significant diet effect ($F(1,16) = 5.0$, $p < 0.040$), indicating that antioxidant-fed

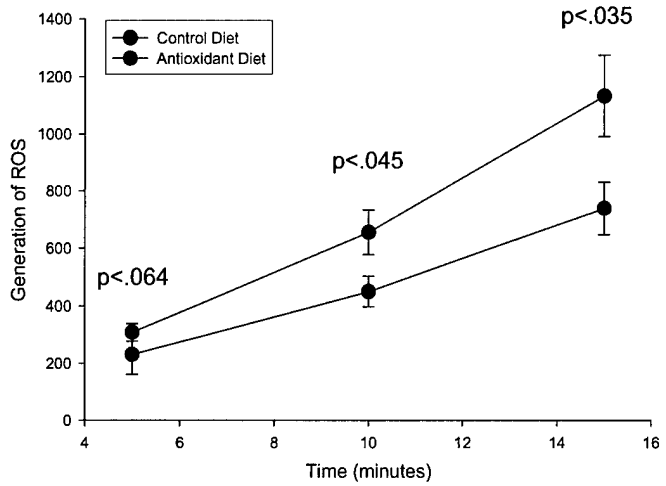

Figure 7. Aged dogs fed a diet rich in antioxidants show less ROS production by isolated mitochondria. The extent of ROS production is plotted as a function of time showing that dogs fed an antioxidant diet show significantly lower ROS production than untreated aged animals. Bars represent standard errors of the mean.

animals showed lower ROS production. A significant time-by-diet interaction ($F(2,32) = 5.62$, $p < 0.008$) reflected larger group differences with increasing time (Figure 7). A similar analysis was used to compare animals receiving behavioral enrichment to those in the control condition. Although similar with respect to time [i.e., all animals showed increasing ROS production with time ($F(2,32) = 73.79$, $p < 0.0001$)], no treatment effect nor significant interactions were observed.

The extent of protein oxidation within isolated mitochondria varied as a function of treatment condition. In the analysis that included the young untreated controls in the ANOVA, significant treatment effects were observed ($F(4,22) = 9.56$, $p < 0.0001$). These effects reflect significantly lower levels of mitochondrial protein oxidation in the young animals as compared to all other treatment groups except the enriched environment only animals (Figure 8). Further, within the aged group of animals, dogs treated with the diet rich in antioxidants and the behavioral enrichment had significantly higher levels of protein carbonyl formation than those receiving the behavioral enrichment alone ($p < 0.028$). To determine overall effects of the diet on protein oxidation, animals receiving the antioxidant diet were compared to those receiving the control diet. The animals provided with the antioxidant diet showed significantly higher protein oxidation in isolated mitochondria ($t(16) = 2.91$, $p < 0.01$). In contrast, comparing animals receiving the behavioral enrichment to those without behavioral enrichment yielded no differences ($t(16) = 0.023$, $p < 0.982$). Protein damage within the mitochondria was not correlated with the extent of ROS production.

Summary: A unique study was implemented to evaluate the function of live, isolated mitochondria from 20 aged animals that were treated for a period of over 2.5 years. ROS production was significantly higher in aged animals relative to young control animals, suggesting impaired mitochondrial function with age. Mitochondrial dysfunction can be partially reversed by providing aged animals with a diet rich in antioxidants and mitochondrial co-factors. In animals receiving the behavioral enrichment protocol alone, higher levels of ROS production were observed relative to untreated aged animals. The extent of protein carbonyl formation in mitochondria was significantly elevated in aged animals as compared to young animals. Somewhat paradoxically, a higher level of oxidative damage to proteins was observed in aged animals provided with dietary antioxidants. These results suggest that either one or combination of the dietary antioxidants improved mitochondrial function, possibly by increasing efficiency (reduction of ROS). However, associated increases in protein oxidation in these same

samples of mitochondria showing reduced ROS may suggest the rapid sequestration of ROS by proteins and subsequent oxidation. These events may also be independent. However, the results may also suggest that further refinement of the dietary components may be indicated in future studies to reduce protein oxidation.

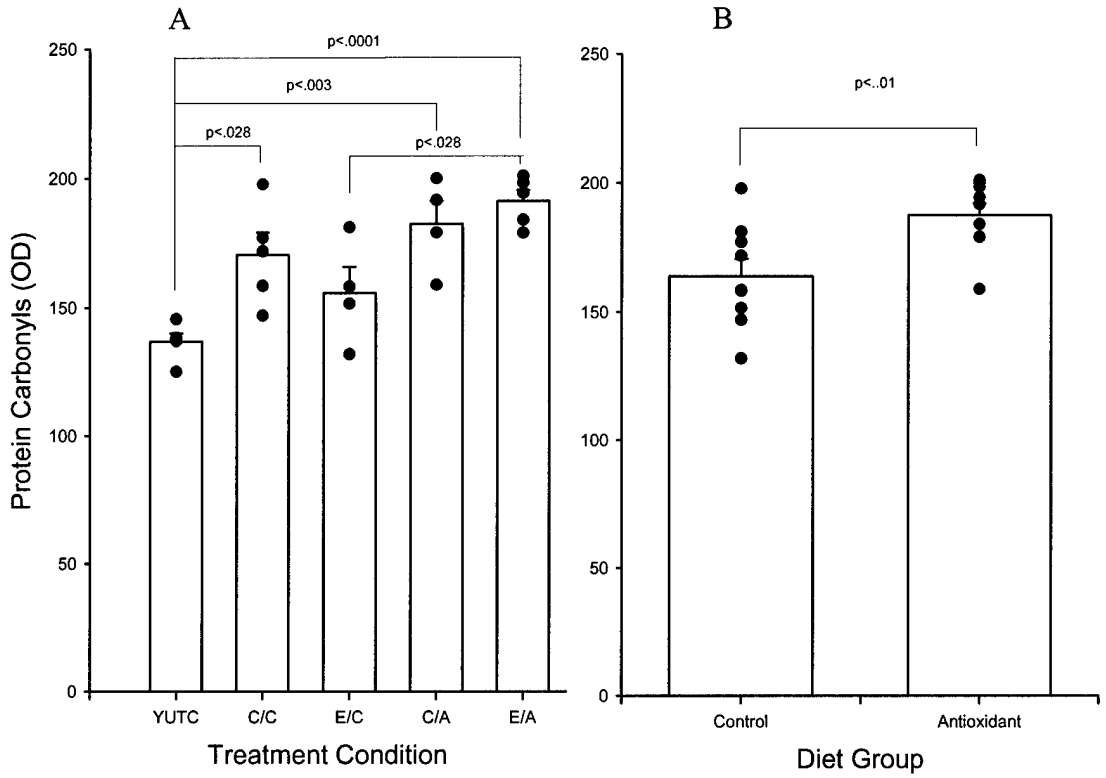

Figure 8. Protein oxidation within isolated canine mitochondria varies as a function of age and treatment. A. Protein carbonyl measures are plotted for individual animals, showing that protein oxidation is increased in aged dogs relative to young dogs. B. In addition, dogs receiving the antioxidant-rich diet show significantly higher protein carbonyl accumulation than untreated aged dogs. Bars represent means, and error bars represent standard errors of the mean. C/C = control enrichment/control diet, E/C = cognitive enrichment/control diet, C/A = control enrichment/antioxidant diet, E/A = behavioral enrichment/antioxidant diet.

F. Mitochondrial Enzyme Function Is Reduced in Aged Animals and Is Partially Reversed by Treatment with a Diet Rich in Antioxidants and Mitochondrial Co-Factors

As mentioned in the previous section, we hypothesized that the addition of the mitochondrial co-factors and cellular antioxidants to the canine senior diet would reduce age-associated mitochondrial dysfunction. A number of enzymes are involved with mitochondrial respiration and are critical to the function of mitochondria. Thus, the function of individual enzymes (complexes I-V) was assayed in live mitochondria. These studies were not proposed in our initial experiments but were incorporated as the expertise became available.

Methods: The cortical biopsy procedure and mitochondrial isolation methods were described in the previous section. Mitochondrial oxygen consumption was measured using a Clark-type electrode in a continuously stirred, sealed chamber (Oxygraph System, Hansatech Instruments Ltd., King's Lynn, U.K.) at 37°C. Isolated mitochondrial protein (500 μg) was suspended in respiration buffer (250 mM sucrose, 20 mM HEPES, 2 mM $MgCl_2$, 2.5 mM inorganic phosphates, 0.1% BSA, pH 7.2) in a final volume of 0.5 ml. State II respiration was initiated by the addition of either 5 mM pyruvate/2.5 mM malate (complex I driven-respiration) or 10 mM succinate (complex II-driven respiration). State III respiration was initiated by the addition of 150 nmol ADP and terminated by the addition of 1 μg/ml oligomycin to the chamber to assess State IV oxygen consumption. State V respiration (maximum oxygen consumption) was achieved by adding the uncoupling agent FCCP (1 μM).

Results: A repeated-measures ANOVA was used to compare mitochondrial enzyme function between young animals (n = 5) and aged untreated controls (n = 5). There was a significant effect of age overall ($F(1,8) = 37.3$, $p < 0.001$) and an age group-by-enzyme interaction ($F(4,32) = 37.2$, $p < 0.001$) (Figure 9). These effects are primarily due to young dogs showing higher activity in complex I and lower activity in complex IV (data not shown).

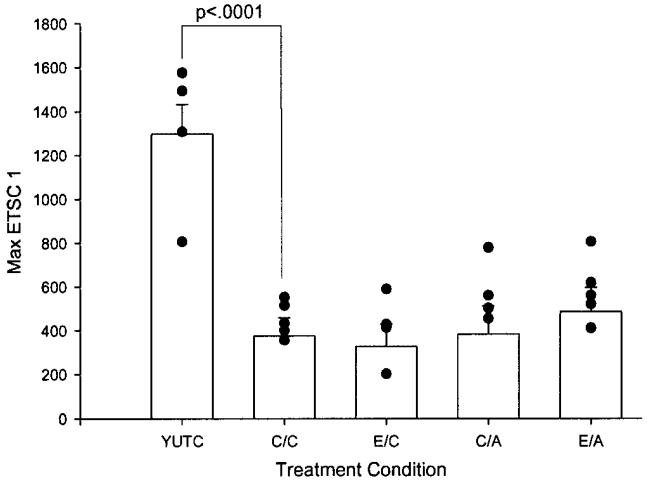

Figure 9. Mitochondrial complex I activity decreases with age and increases in response to treatment. Individual mitochondrial complex I activity is plotted as a function of age and treatment condition. When all four treatment groups are considered, there are no significant effects. Bars represent means, and error bars represent standard errors of the mean. C/C = control enrichment/control diet, E/C = cognitive enrichment/ control diet, C/A = control enrichment/antioxidant diet, E/A = behavioral enrichment/antioxidant diet.

A repeated-measures ANOVA was also used to compare different treatment conditions within the aged group of dogs on measures of mitochondrial function. Post hoc comparisons were done using Bonferroni corrections or by Dunnett's t-test using the aged untreated animals as the control group. Overall there was no main effect of the treatment ($F(3,14) = 1.74$, $p < 0.204$), but the interaction between which mitochondrial complex was measured and treatment group approached significance ($F(12,56) = 1.87$, $p < 0.058$) (Figure 9). This effect appeared to be due to the two antioxidant treatment groups appearing more

similar than either the untreated aged controls or the behavioral enrichment group. Thus, a comparison was made between the dogs receiving the antioxidant diet versus those receiving the control diet. A significant main effect of diet was observed ($F(1,16) = 5.27$, $p < 0.036$) as well as a significant diet effect-by-mitochondrial enzyme interaction ($F(4,64) = 6.1$, $p < 0.0001$). Antioxidant-treated dogs showed higher mitochondrial complex I activity than the dogs fed the control senior diet ($t(16) = 2.47$, $p < 0.025$) (Figure 10).

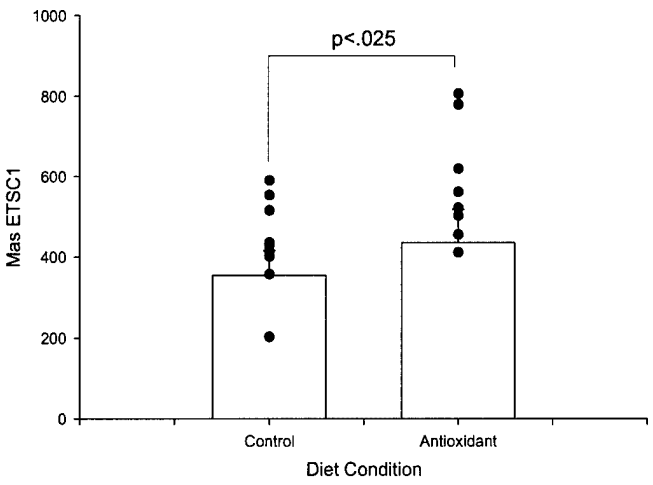

Figure 10. Mitochondrial complex I activity increases in response to antioxidant treatment. Individual mitochondrial complex I activity is plotted as a function of diet for the animals in the control diet group versus the antioxidant diet (behavioral enrichment is not considered in this analysis). Dogs receiving the antioxidant diet show significantly higher mitochondrial complex I activity than untreated controls. Bars represent means, and error bars represent standard errors of the mean.

Summary: Significant age and treatment effects were observed for mitochondrial enzymes. In particular, complex I, the first component of the mitochondrial complex chain exhibited a significantly higher level of activity in young animals compared to old animals. The activity of this enzyme was improved in aged animals provided with a diet rich in a broad spectrum of cellular antioxidants as well as mitochondrial co-factors. Thus, the addition of mitochondrial co-factors either alone or in combination with the cellular antioxidants may have resulted in improved mitochondrial function by increased complex I activity.

G. Lipid Peroxidation as a Function of Age and Treatment in Various Neocortical Regions of the Brain

We previously reported age-dependent increases in lipid peroxidation measured by the accumulation of malondialdehyde (MDA) [12]. In that study we also observed a significant correlation between brain MDA and serum MDA, suggesting a possible method to evaluate ongoing effects of an antioxidant treatment trial. The actual source of MDA is difficult to establish because aldehydes can reach targets distant from the original site of oxidation [13]. An alternative, and more likely, explanation is that serum MDA is derived from lipid damage in both central and peripheral systems. Experiments used to modify peripheral oxidative stress with dietary antioxidants result in both improved peripheral and central measures [14]. Further, the

link between peripheral and central measures of lipid peroxidation we reported in aged canines suggests that serum MDA may be a useful endpoint measure to monitor antioxidant interventions *in vivo*.

In previous progress reports we described data showing that MDA in plasma was increased in the dogs provided with environmental enrichment and significantly reduced in one group of animals (Hill's beagles) provided with the antioxidant diet. We have now measured MDA in several cortical regions in the treated and untreated animals to determine whether the antioxidant diet reduced lipid peroxidation in the central nervous system.

Methods: At the time of brain tissue harvesting, samples from the prefrontal, occipital, parietal, and temporal cortex were dissected and flash frozen for biochemical assays. As described in the previous report, these samples were shipped to Drs. Liu and Ames at the University of California-Berkeley in a collaborative study.

Cortical samples were prepared in 10 vol of homogenizing buffer (100 mM Tris, pH 8.0, 100 mM NaCl, 20 mM EDTA) with proteinase inhibitors (leupeptin 0.5 μg/ml, apoprotonin 0.5 μg/ml, pepstatein 0.7 μg/ml). The presence of EDTA was intended to reduce the potential for Fe oxidation. Phenylmethylsulfonyl fluoride was added at 40 μg/ml just before homogenizing. The protein-bound MDA was hydrolyzed with H_2SO_4. MDA was converted to a stable derivative using pentafluorophenyl hydrazine at room temperature. The derivative was extracted with isooctane and detected with a Hewlett Packard 5890 Series II gas chromatograph interfaced to a 5989 mass spectrometry system equipped with a J&W Scientific DBWAX capillary column (15 m × 0.25 mm i.d., 0.25 μm film thickness) in the negative chemical ionization mode [15]. The results were indexed to protein, which was measured using a microtiter plate assay with bicinchoninic acid (BCA) kit from Pierce (Rockford, IL).

Results: Figure 11 shows the differential effects of age and treatment on the four brain regions sampled. A repeated-measures ANOVA indicates a significant overall effect of the cortical region sampled, with the prefrontal cortex showing the highest levels of lipid peroxidation and the occipital cortex showing the lowest. The parietal and temporal cortices were intermediate in the extent of lipid peroxidation.

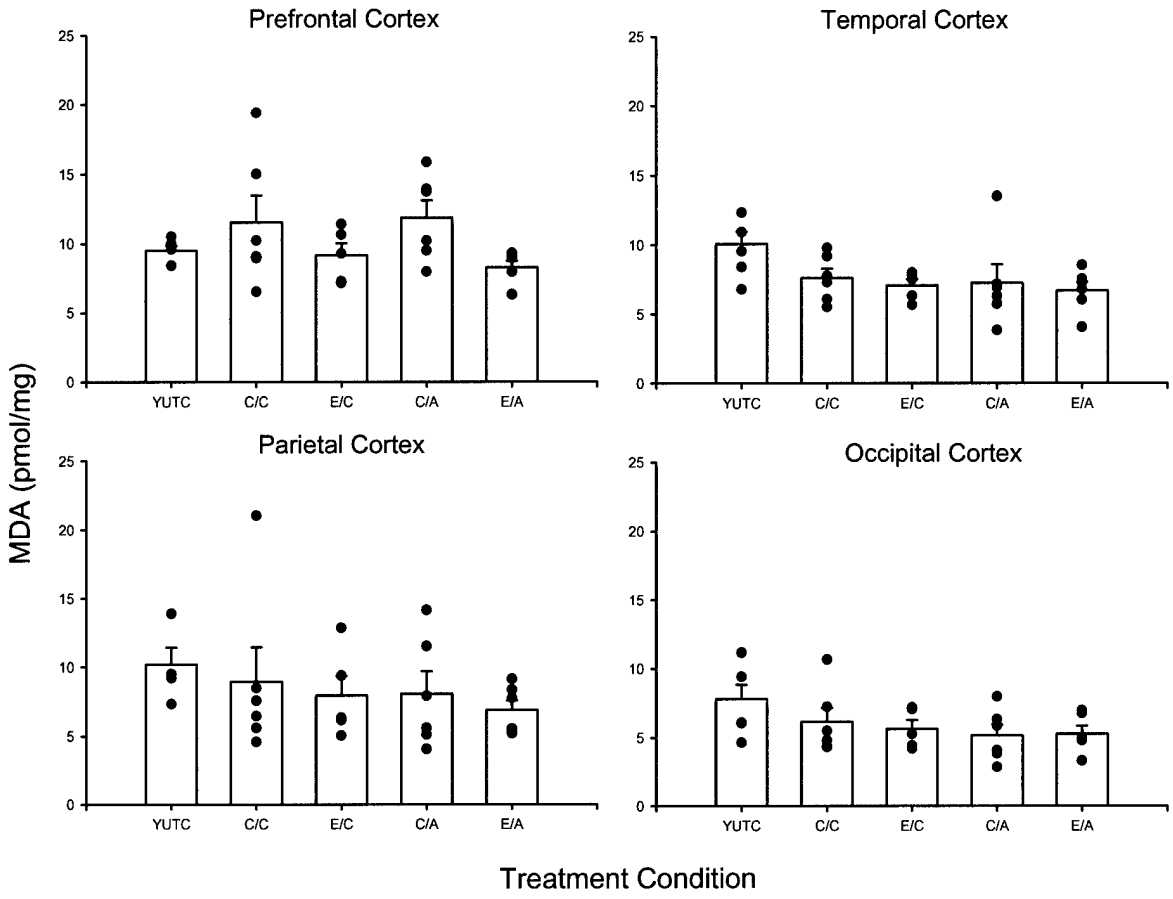

Figure 11. Accumulation of malondialdehyde (MDA), a product of lipid peroxidation in four different cortical regions does not vary as a function of treatment. Each brain region is plotted separately and shows the individual dog MDA values separated into treatment groups. Bars represent means, and error bars represent standard errors of the mean. C/C = control enrichment/control diet, E/C = cognitive enrichment/control diet, C/A = control enrichment/antioxidant diet, E/A = behavioral enrichment/antioxidant diet.

No significant age effects overall were observed when comparing the five young animals with five aged untreated controls. This is in contrast to our previous publication showing significant age effects in a larger group of animals (n = 19) ranging in age from 4.5–15.3 years [12]. There were also no significant treatment effects using separate ANOVAs in each brain region. Overall, however, the combined treatment group showed lower levels of lipid peroxidation in the parietal and prefrontal cortex than the other treatment conditions.

Summary: The results suggest that neither the antioxidant diet nor the behavioral enrichment had a significant impact on the accumulation of one lipid peroxidation product, malondialdehyde, in any of the cortical regions sampled. Thus, the antioxidant diet may have had little effect on lipid peroxidation in the central nervous system (CNS) despite some small but

clear decreases in peripheral measures we reported previously. This suggests that plasma and serum measures of MDA primarily reflect antioxidant treatment effects on peripheral systems.

H. Protein Oxidation and Enzyme Function in the Brain as a Function of Age and Treatment

In a previously published report, we demonstrated that the extent of protein oxidation and level of glutamine synthetase activity increase and decreased, respectively, as a function of age [12]. Thus, we measured these same two markers of oxidative damage in the current study and hypothesized that animals fed an antioxidant diet would show reduced protein carbonyl formation and higher glutamine synthetase activity than animals fed the control diet.

At the time of brain tissue harvesting, samples from the prefrontal, occipital, parietal, and temporal cortex were dissected and flash frozen for biochemical assays. As described in the previous report, these samples were shipped to Drs. Liu and Ames at the University of California-Berkeley in a collaborative study. A total of 5 young animals (< 5 years) and 22 aged animals were available for study. Two aged animals were not included because one died prior to the start of intervention and the second was euthanized prior to 2 years of being on treatment.

***Methods*:**

Protein Carbonyl Assay. Frozen prefrontal cortex homogenates were used to measure the protein carbonyl content by labeling protein hydrazone derivatives using 2,4-dinitrophenylhydrazide (DNPH) according to the method of Levine [16].

Glutamine Synthetase (GS) Activity. GS activity in the prefrontal cortex was determined using the technique described by Rowe et al. [17]. Corrections were made for nonspecific glutaminase activity by comparing total activity in the presence and absence of adenosine diphosphate and arsenate.

***Results*:** To test for age effects, five young animals were compared to the six untreated aged controls. In this limited sample, there was no significant difference in protein carbonyl accumulation nor in glutamine synthetase activity (Figures 12 and 13, respectively). In the temporal cortex, however, the young animals had higher glutamine synthetase activity, with the statistical results approaching significance ($t(9) = 1.92$, $p < 0.087$). Two repeated-measures ANOVAs were used to test for treatment effects across cortical regions for each marker of oxidative damage separately. Overall, differences in the amount of protein carbonyl formation

varied as a function of brain region ($F(3,51) = 2.53$, $p < 0.067$). No significant treatment effects were observed for protein carbonyl formation. For GS activity, no significant brain region differences were observed. However, a significant treatment effect ($F(3,18) = 3.17$, $p < 0.05$) indicated that the untreated aged animals had lower GS activity overall. Much of this effect was due to decreased activity in the prefrontal and temporal cortex. A second analysis involved collapsing the groups down to compare animals fed the antioxidant diet versus those fed the control diet. None of the groups were significantly different on measures of protein carbonyls nor GS activity.

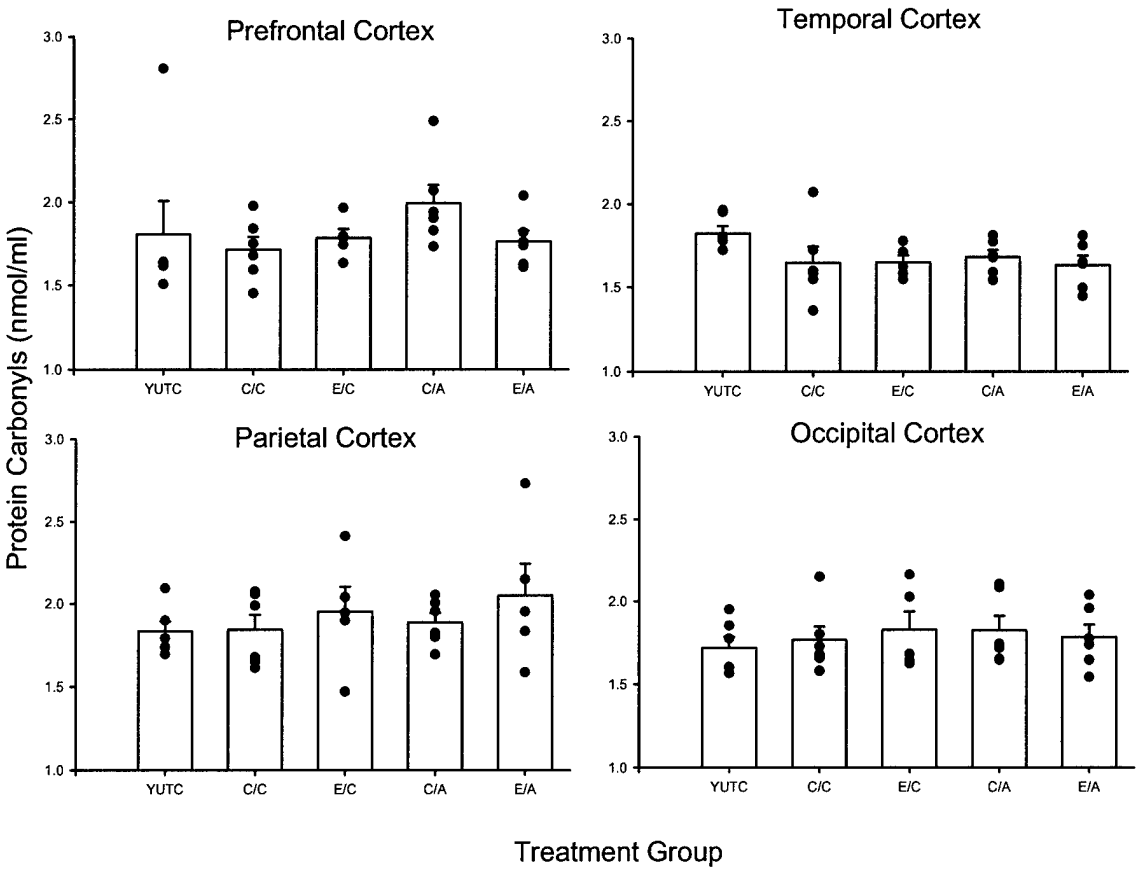

Figure 12. Protein carbonyl accumulation in the brain does not vary as a function of treatment. Each brain region is plotted separately and shows the individual dog protein carbonyl values separated into treatment groups. Bars represent means, and error bars represent standard errors of the mean. C/C = control enrichment/control diet, E/C = cognitive enrichment/control diet, C/A = control enrichment/antioxidant diet, E/A = behavioral enrichment/antioxidant diet.

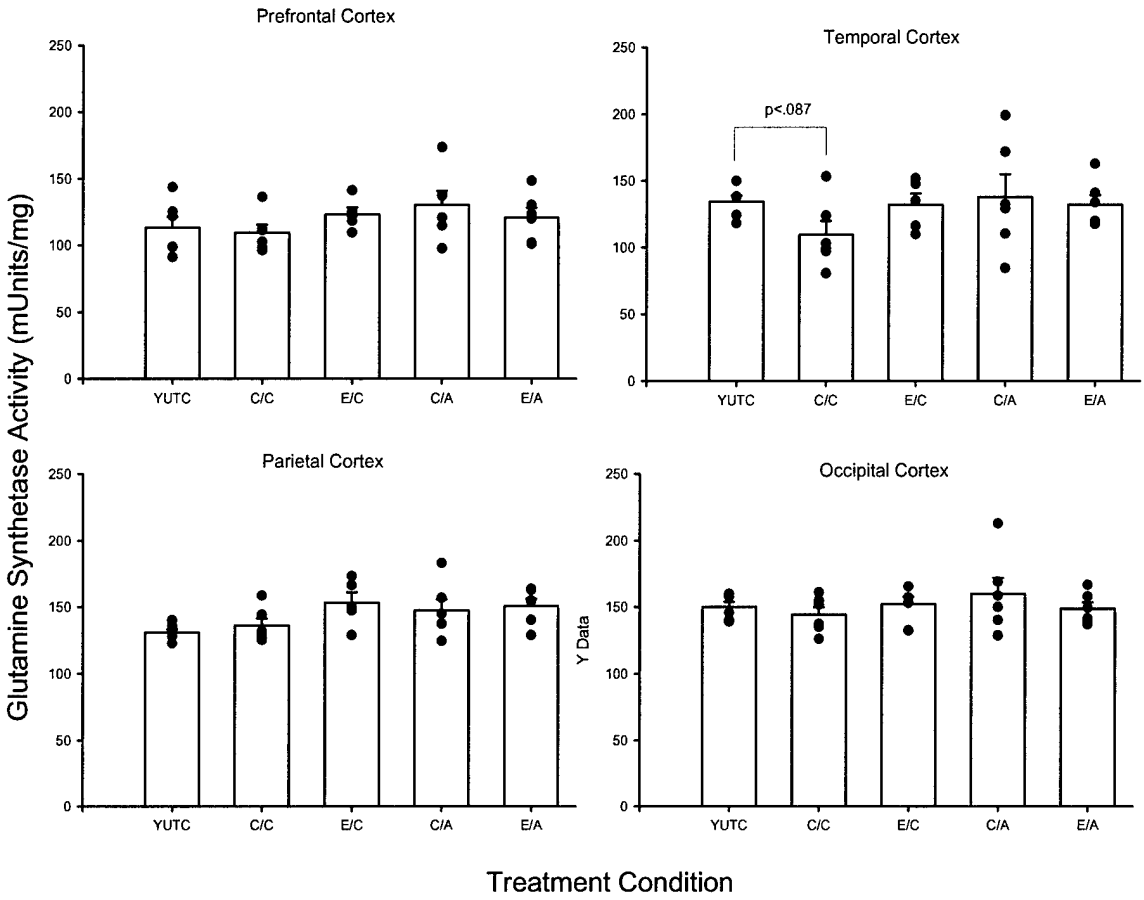

Figure 13. Glutamine synthetase (GS) activity, another measure of protein oxidation, as a function of age and treatment in the canine brain. Each brain region is plotted separately and shows the individual dog protein carbonyl values separated into treatment groups. Note that in the temporal cortex, young animals have higher GS activity than aged, untreated animals. Bars represent means, and error bars represent standard errors of the mean. C/C = control enrichment/control diet, E/C = cognitive enrichment/control diet, C/A = control enrichment/antioxidant diet, E/A = behavioral enrichment/antioxidant diet.

Summary: The cortical levels of protein oxidation (protein carbonyls) and consequent enzyme dysfunction (GS) were measured in the prefrontal, temporal, parietal, and occipital cortices in 5 young and 22 treated aged animals. Age effects were not observed in either endpoint measure except for higher GS activity in the temporal cortex of young animals. The extent of protein carbonyl formation varied as a function of brain region but did not change in response to treatment. GS activity showed little variation across brain region but was sensitive to the treatment condition. Animals in the untreated control group showed significantly lower levels of enzyme activity than any of the treatment groups, particularly in the temporal and prefrontal cortex. When these results are compared with the lack of effect on MDA accumulation in the brain, one possible interpretation is that the antioxidant diet may have had a larger impact on protein oxidation than on lipid peroxidation.

I. Terminal Study Measures of Brain, CSF, and Plasma Isoprostanes as a Function of Age and Treatment

There are many measures of lipid peroxidation, but one in particular, isoprostane (isoP) , reflects both the central and peripheral accumulation of oxidative damage. The rationale for measuring isoP includes: (1) isoPs are specific products of lipid peroxidation; (2) they are stable compounds; (3) levels of isoP are detectable in many tissue types; (4) the formation of isoP is modulated by antioxidant status; and (5) isoP level is unaffected by lipid content of the diet [18]. In addition, several studies in both mice and in humans show that this marker of lipid peroxidation is sensitive to age and disease [19–21].

In our previous report we provided preliminary data describing the results from one measure of lipid peroxidation conducted in plasma samples from dogs in the study and the results of a study of age effects on several other measures of oxidative damage [12]. In the progress report, we show that MDA in the brain remains unchanged in the antioxidant-treated groups. Thus, although not proposed in our initial experiments, the measurement of isoP in the brain was subsequently incorporated.

***Methods*:** At the terminal phase of the study, plasma and cerebrospinal fluid (CSF) were drawn from animals, aliquoted, and used for isoP assays. In addition, a section of temporal cortex was dissected and frozen at –80° until assay. Of the 24 aged animals from LRRI that began the study, 21 survived to the end of treatment and were maintained for a period of over 2.5 years. The amount of F_2-isoP (ng/mg) was determined by gas chromatography/negative chemical ionization mass spectrometery as described previously [22].

***Age effects*:** In a comparison between 5 untreated young control animals and 21 old animals that survived the full study, the plasma level of isoP was significantly higher in the young dogs ($t(22) = 2.6$, $p < 0.015$; $n = 5$ young, $n = 19$ aged) (Figure 14). There were insufficient CSF samples from young animals to make age comparisons in these tissues (Figure 15, note: aged animals tend to show enlarged ventricles and thus larger volumes of CSF that can be drawn relative to young animals). In the temporal cortex, the opposite trend was observed, and differences in the level of isoP were lower in young animals compared to all the old animals ($t(25) = 1.87$, $p < 0.073$) (Figure 16).

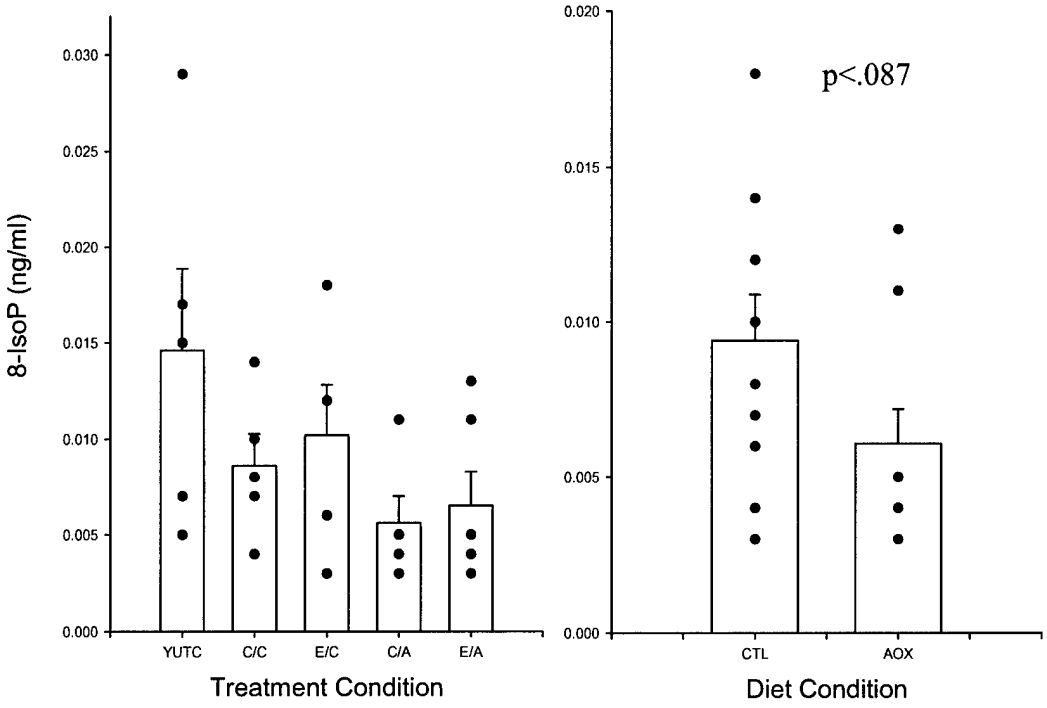

Figure 14. Plasma lipid peroxidation, measured by the accumulation of isoprostanes (isoP), is decreased in treated aged animals. Individual isoP levels are plotted as a function of age and treatment. A large amount of individual variability is observed in young animals. Aged animals provided with a diet rich in antioxidants show less accumulation of isoP in plasma. Bars represent means, and error bars represent standard errors of the mean. C/C = control enrichment/control diet, E/C = cognitive enrichment/control diet, C/A = control enrichment/antioxidant diet, E/A = behavioral enrichment/antioxidant diet. Ctl = control diet, Aox = antioxidant diet.

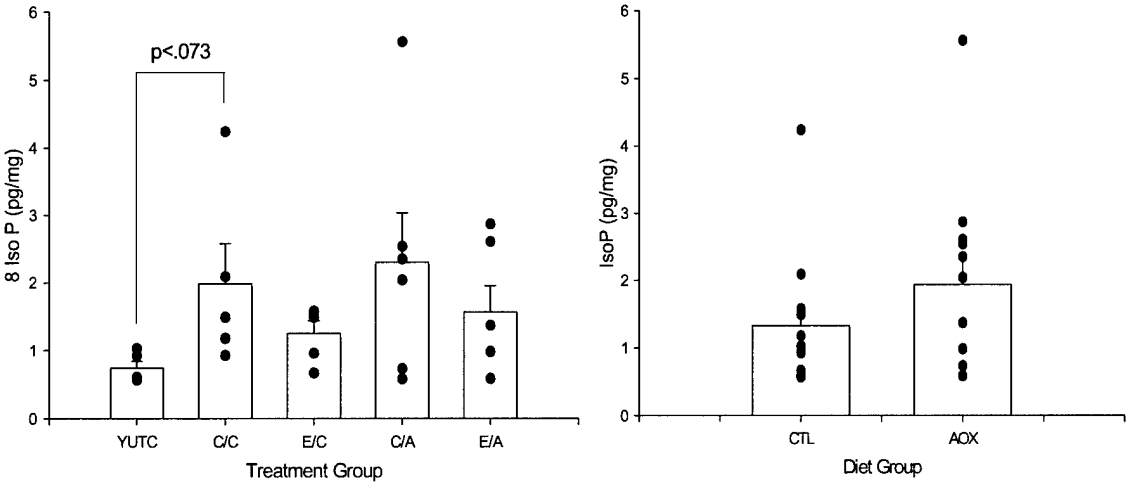

Figure 15. Cerebrospinal fluid lipid peroxidation, measured by the accumulation of isoP, is increased in treated aged animals. Individual isoP levels are plotted as a function of age and treatment. Young animals appear to have lower CSF isoP than aged animals, but the sample size limited the statistical analysis. Aged animals provided with a diet rich in antioxidants show higher levels of isoP in CSF. Bars represent means, and error bars represent standard errors of the mean. C/C = control enrichment/control diet, E/C = cognitive enrichment/control diet, C/A = control enrichment/antioxidant diet, E/A = behavioral enrichment/antioxidant diet.

Treatment effects: Terminal plasma assay of isoP indicated no overall treatment effects in aged dogs. However, dogs fed the antioxidant diet had marginally significantly lower isoP in plasma than control diet fed animals ($t(19) = 1.8$, $p < 0.087$) (Figure 14). In CSF (Figure 15), there were no significant treatment effects within the group of aged animals ($F(3,13) = 1.63$, $p < 0.243$). There was a general trend toward higher levels of CSF isoP in antioxidant-treated dogs ($t(12) = 1.87$, $p < 0.086$). In the temporal, cortex no treatment effects were noted nor were clear differences between the antioxidant fed dogs and the control animals observed (Figure 16).

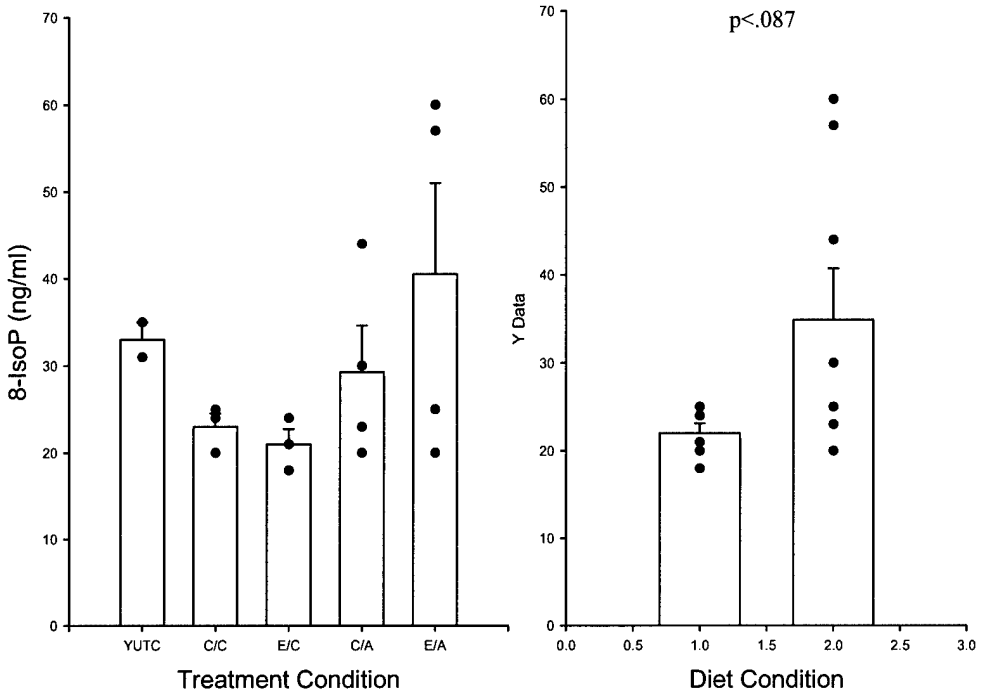

Figure 16. Brain lipid peroxidation, measured by the accumulation of isoP, is increased in aged animals but remains relatively unchanged in treated animals. Individual temporal cortex isoP levels are plotted as a function of age and treatment. Aged animals show higher amounts of accumulated isoP in the temporal cortex relative to young animals. The antioxidant diet did not significantly reduce brain lipid peroxidation. Bars represent means, and error bars represent standard errors of the mean. C/C = control enrichment/control diet, E/C = cognitive enrichment/control diet, C/A = control enrichment/antioxidant diet, E/A = behavioral enrichment/antioxidant diet.

Summary: Accumulation of lipid peroxidation production varied as a function of age, with young animals showing lower levels than old animals in plasma and in the temporal cortex. IsoP appeared lower in peripheral samples (i.e., plasma) but marginally higher in CSF samples in antioxidant fed aged dogs. No difference in temporal cortex accumulation of isoP was observed in response to the antioxidant diet. The lack of effect of the antioxidant diet on lipid peroxidation was thus confirmed using a second marker in an independent research laboratory. However, as with MDA, peripheral assays were more sensitive to age and treatment

in the current experiments. These results combined suggest that whereas systemic lipid peroxidation may vary with age and treatment, brain levels remain unchanged in response to treatment. These results raise the possibility that serum or plasma lipid peroxidation may provide sufficient samples to assay and monitor antioxidant treatment effects of peripheral but not central systemic response to treatment.

J. Aβ Accumulation Is Significantly Reduced in Antioxidant Diet Treatment Group

Previous studies associate oxidative damage with Aβ accumulation. When applied in cell culture systems, Aβ can lead to oxidative damage by inducing the formation of oxygen and nitrogen-reactive species and depleting endogenous antioxidants [23–25]. *In vitro* application of specific antioxidants, such as alpha-tocopherol, can protect neurons against Aβ-mediated toxicity without directly reducing the oxidative stress produced by Aβ [26]. Correlative anatomical studies in human brains demonstrate the colocalization of Aβ with markers of oxidative damage to proteins, lipids, and DNA [27–30]. Other evidence suggests that oxidative damage occurs prior to Aβ accumulation and may even enhance it because oxidation of the Aβ precursor protein leads to the increased production of amyloidogenic fragments [31–35]. Consistent with this hypothesis are observations of a rise in lipid peroxidation prior to Aβ accumulation in transgenic mice [19] and the reduction of Aβ-induced cognitive deficits and neuropathology in mice fed the antioxidant, curcumin [36].

***Methods*:** Twenty minutes before induction of general anesthesia, animals were sedated by subcutaneous injection with 0.2 mg/kg acepromazine. General anesthesia was induced by inhalation with 5% isoflurane. While being maintained under anesthesia, dogs were exsanguinated by cardiac puncture, and blood samples were collected to obtain plasma and serum for future studies. Within 15 minutes, the brain was removed from the skull and a CSF sample was obtained from the lateral ventricles. The brain was sectioned midsagitally with the entire left hemisphere being immediately placed in 4% paraformaldehyde for 48–72 hours at 4°C prior to long-term storage in phosphate buffered saline with 0.05% sodium azide at 4°C. The remaining hemisphere was sectioned coronally and flash frozen at –80°C. The dissection procedure was completed within 20 minutes, thus the post-mortem interval for all animals was 35–45 minutes.

The left hemisphere was embedded in a gelatin matrix (Neuroscience Associates, Knoxville, TN), serial coronal sections were cut at 40 μm through the entire hemisphere, and the sections were placed in antigen preserve solution [phosphate buffered saline, ethylene glycol, polyvinylpyrrolidine (PVP)] at –20°C. Free-floating sections containing four regions of interest were selected from the coronal sections. The rationale for selecting these specific regions was that they were components of cortical circuits responsible for intact or impaired function on cognitive tasks used in the study. Second, these same regions were described in a previous study, establishing the pattern of Aβ deposition as a function of age in canines [37]. These regions included the dorsolateral prefrontal cortex, the entorhinal cortex, the posterior parietal cortex, and the occipital cortex [38]. For the dorsolateral prefrontal cortex section, we used the dorsal aspect at the anterior tip of the brain called the "proreus" or "prorealis" [38]. The entorhinal cortex section was analyzed midway between the rostral and caudal poles of the hippocampus. The parietal cortex section was sampled from the same coronal section as the entorhinal cortex. For the parietal cortex, we used the most dorsal aspect of the brain corresponding to area marginalis posterior [39]. Finally, the occipital cortex section was derived from the most posterior coronal section of the brain, and the most dorsal aspect corresponding to area marginalis posterior was used for analysis [38]. The occipital section is also called the "area marginalis posterior" but was much further posterior than the area taken from the parietal cortex. Two sections from each cortical region were used and were separated by at least 200 μm.

Aβ was detected with anti-Aβ1-17 (6E10 antibody, mouse monoclonal human anti-Aβ1-17, 1:5000, Signet Laboratories, Inc., Dedham, MA). Sections were pretreated for 4 minutes with 90% formic acid [40] prior to overnight incubation at room temperature in 6E10 in Tris-saline with 2.0% bovine serum albumin and 0.1% Triton X-100. Bound secondary antibody was detected using anti-mouse ABC peroxidase kits from Vector Laboratories (Burlingame, CA). Aβ immunostaining was visualized using a DAB substrate kit from Vector Laboratories. Control experiments where primary or secondary antibody was omitted resulted in negative staining.

The procedure for obtaining Aβ loads has been reported previously [37]. Aβ immunostaining was captured using a 2.5 photo eyepiece, a Sony high-resolution CCD video camera (XC-77), the built-in video capture capabilities of a Macintosh 8100/80AV, and NIH Image 1.59b5. For every animal, four brain regions were outlined, and ten images (525 ×

410 μm each) were captured from each brain region at a 20X objective. Sampling consisted of five images from the superficial cortical layers, and five from the deep cortical layers (for a total of 40 images per animal × 24 study dogs = 960 samples, and 40 images × 5 young dogs = 200 samples). The cross-sectional area occupied by Aβ in each individual image was quantified using gray-scale thresholding. This thresholding procedure separates positive staining from background and calculates the percentage of area occupied by Aβ immunoreactivity.

To preclude bias, Aβ characterization (load analysis, typing, and angiopathy quantification) were collected while blind with respect to the experimental conditions. To determine whether any of the four treatments affected the extent of Aβ load deposition in the aged canine brain, a repeated-measures ANOVA was used. The proportion of area occupied by Aβ was converted to a log10 score to reduce variability across treatment groups and satisfy the assumptions for an ANOVA. The mean of the log scores were subsequently used in all statistical analyses. When appropriate, independent t tests were also used. SPSS for Windows (SPSS, Inc., Chicago, IL) or SAS (SAS Institute, Inc., Cary, NC) was used for all statistical analyses.

Results: Of the 24 aged animals that began the study, 4 required euthanasia during the study (as indicated in Table 1). One animal required euthanasia during the baseline period and was not included in any of the anatomical studies. Twenty-three animals were treated for an average of 2.69 years (SD = 0.19) and were included in the final data analysis. There were no significant differences across the four groups in terms of the average age of animals at the end of the study ($F(3,22) = 1.13$, $p = 0.36$) or the duration of the treatment protocol ($F(3,22) = 0.32$, $p = 0.81$). Five animals described in previous sections were included as young untreated controls.

Aβ load varied as a function of age, brain region, the cortical layer sampled, and across treatment conditions. As reported previously, Aβ was observed only within the aged group of animals and little or no Aβ was detected in young dog brains. Figure 17 shows Aβ immunostaining from an aged animal from each treatment group to illustrate both the treatment and brain region differences. The majority of this effect was due to differences in the accumulation of Aβ in the deep cortical layers and not in the superficial cortical layers. Similar effects were obtained in a replication experiment with a second section from each cortical region (data not shown). A repeated-measures ANOVA comparing treatment effects on Aβ loads from

the deep cortical layers across the four brain regions indicated a significant effect of the antioxidant diet overall ($F(1,19) = 5.295$, $p = 0.033$) (Figures 18A and 18B) but not for behavioral enrichment ($F(1,19) = 0.001$, $p = 0.982$) (Figure 19). The interaction between the two treatments was not significant ($F(1,19) = 0.008$, $p = 0.929$). Although Aβ varied as a function of cortical region sampled ($F(3,57) = 2.71$, $p = 0.053$), the interaction between diet and region sampled was not significant ($F(3,57) = 0.783$, $p = 0.508$).

When the ANOVA is repeated placing animals into two groups based on either receiving the antioxidant diet or the control diet, regardless of behavioral enrichment, these effects become larger ($F(1,21) = 5.86$, $p = 0.025$) (Figure 18B). Despite a lack of interaction between diet and cortical region sampled, plots of the data suggested differential treatment effects (Figure 2). Thus, separate independent t tests were used to test for diet effects within each brain region. Treatment with an antioxidant diet did not significantly reduce Aβ in the prefrontal ($t(21) = 1.438$, $p = 0.165$) nor in the occipital cortex ($t(21) = 1.194$, $p = 0.246$). However, animals provided with an antioxidant diet showed significant lower Aβ accumulation in the entorhinal ($t(21) = 2.166$, $p = 0.043$) and parietal ($t(21) = 2.137$, $p = 0.046$) cortex.

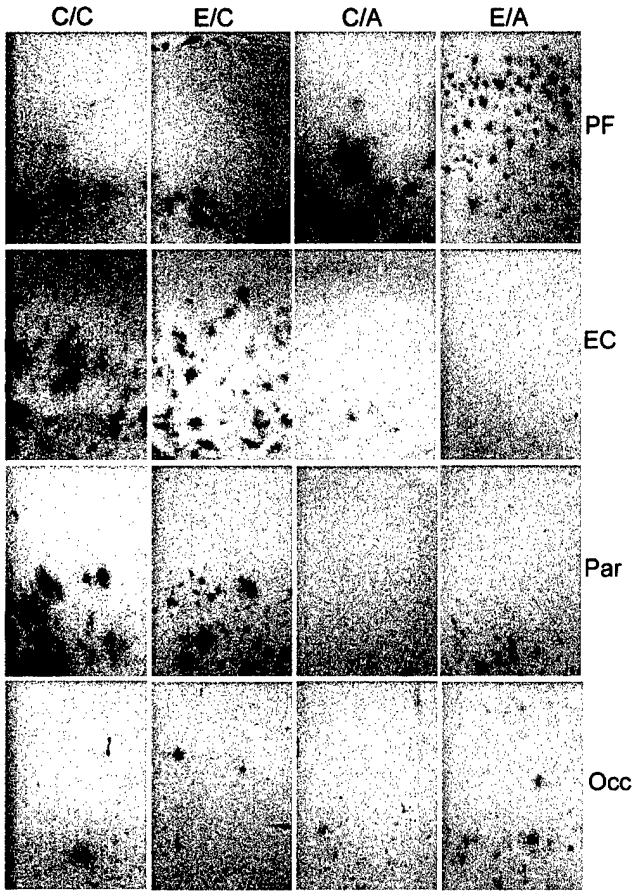

Figure 17. Aβ deposition is reduced in antioxidant-treated animals in the entorhinal and parietal cortex but not the prefrontal nor occipital cortex. A series of images obtained from one animal in each treatment group shows differences in Aβ deposition. C/C = control enrichment/control diet, E/C = cognitive enrichment/control diet, C/A = control enrichment/antioxidant diet, E/A = behavioral enrichment/antioxidant diet. Brain regions: PF = prefrontal cortex, EC = entorhinal cortex, Par = parietal cortex, Occ = occipital cortex.

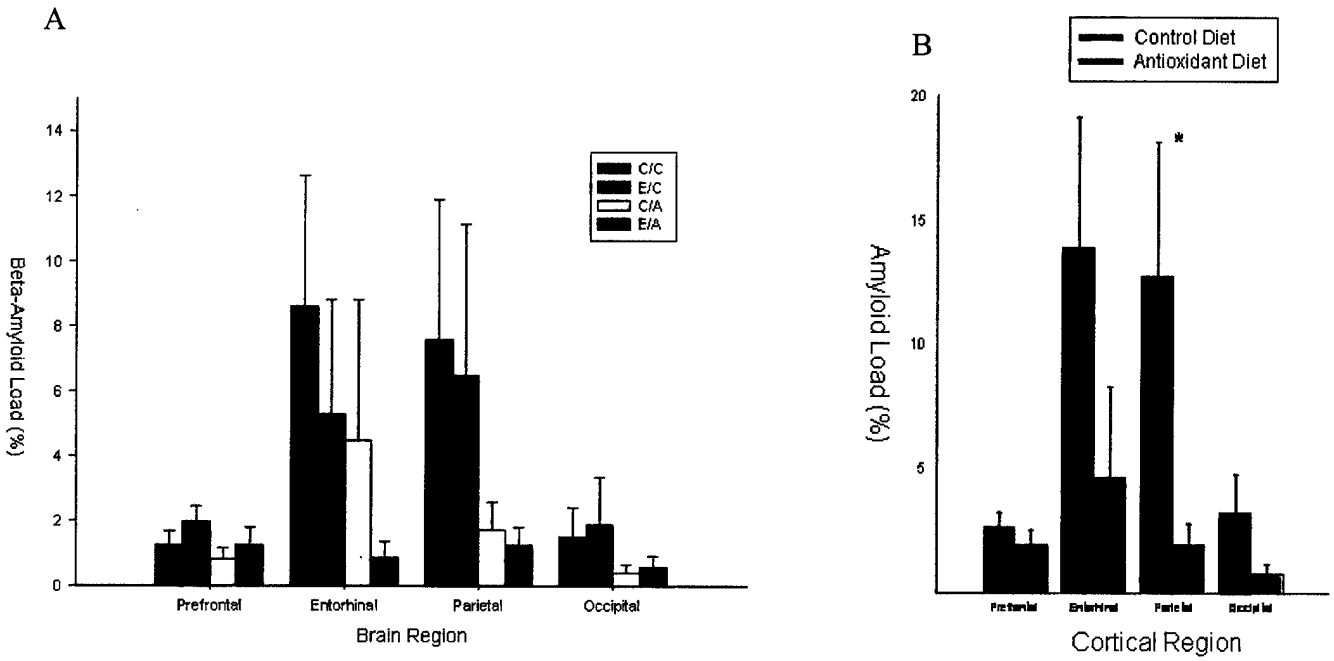

Figure 18. Aβ loads are decreased in specific brain regions in the antioxidant but not behavioral enrichment treatment groups. A. Mean Aβ loads are plotted for each treatment condition for each brain region showing that the antioxidant-treated animals have lower Aβ loads. B. When animals are combined into antioxidant diet or control diet groups only, the differences can be clearly observed in the parietal and entorhinal cortices.

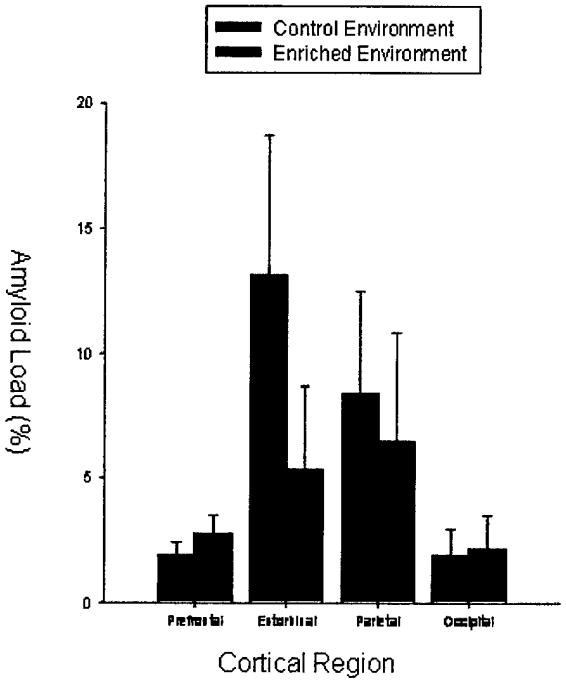

Figure 19. In contrast, the effect is selective for the antioxidant-treated groups as the animals receiving behavioral enrichment do not show reduced Aβ relative to the control enrichment condition. C/C = control enrichment/control diet, E/C = cognitive enrichment/control diet, C/A = control enrichment/antioxidant diet, E/A = behavioral enrichment/antioxidant diet.

Summary: Aβ deposition in the antioxidant-treated animals was lower in the parietal and entorhinal but not in the occipital and prefrontal cortices. In addition, the antioxidant diet considerably reduced individual variability, indicating that all old dogs benefited from the treatment. No Aβ-lowering effects were observed in animals receiving the enriched behavior protocol alone. These results suggest that a diet rich in antioxidants can slow but not reverse Aβ deposition. A new set of experiments has now been designed that will be used to determine the mechanism underlying reduced Aβ accumulation. Studies will examine the role of Aβ turnover (measuring insulin-degrading enzyme and neprilysin level) or changes in amyloid precursor protein processing (proteomics, measuring secretase activity). The dose levels used in the current study are consistent with human clinical trials and indicate that dietary supplementation with a broad spectrum of antioxidants may prove beneficial for human brain aging.

K. Synaptophysin as a Function of Age and Treatment in the Prefrontal Cortex and Hippocampus

One of the hypotheses we had originally proposed was that increasing behavioral enrichment would be associated with increased synapse growth or maintenance of synapses over the aging process [41–43]. To evaluate this hypothesis, both Western blotting and immunohistochemical measures of synaptophysin, which is a protein associated with presynaptic vesicles, was assayed. This endpoint marker was proposed initially for our study but will be expanded over the next year to include other synapse markers (e.g., synaptobrevin, synapsin, etc). Thus, we predicted that synaptophysin levels would be highest in young animals and lowest in untreated aged animals. We expected that the animals receiving the behavioral enrichment protocol would show higher synaptophysin levels than untreated aged controls. The antioxidant diet intervention may also interact with behavioral enrichment to further enhance the survival of neurons and maintenance of synapse numbers.

Methods: 0.15 grams of prefrontal cortex and hippocampus were homogenized in 1.5 ml of extraction buffer [1% SDS, 100 mM Tris, pH 6.8 and protease inhibitor cocktail ICN Cat # 158837 containing 167 mM (0.04%) AEBSF, 26.9 mM (0.1%) di-sodium EDTA, 0.21 mM (0.0001%) leupeptin, and 0.146 mM (0.0001%) pepsatin A]. Homogenates were boiled for 5 minutes prior to centrifugation at 14,000 rpm, three times for 30 minutes. The supernatant was removed and assayed for protein concentration using the Pierce BCA protein assay microwell

plate (Pierce Biotechnology, Inc., Rockford, IL) protocol (spectroscopy), a ThermoMax microplate reader (Molecular Devices Corporation, Sunnyvale, CA) at 570 nm wavelength, and the SOFTmax Version 2.32 FPM software (Molecular Devices Corporation).

10 or 15 micrograms of protein from hippocampus and prefrontal cortex of each dog was separated using 4–12% sodium dodecyl sulfate-polyacrylamide-gel electrophoresis (SDS-PAGE). Proteins were transferred to polyvinylidene difluride (Sequi-Blot PVDF from Bio-Rad Laboratories, Hercules, CA) membranes for immunoblotting. Membranes were blocked overnight in 5% nonfat dry milk in TTBS (10 mM Tris, pH 7.6, 100 mM NaCl, and 0.1% Tween 20) at 4°C. The membranes were incubated in mouse anti-synaptophysin (Chemicon Cat # MAB 5258) 1 mg/ml (1:5000) at room temperature, washed six times for 5 minutes with TTBS, then incubated in goat anti-mouse HRP conjugated at 1:5000 (Bio-Rad Cat # 172-1011) for 1 hour. After six washes of 5 minutes each, proteins were visualized using Supersignal® West Pico Chemoluminescent Substrate from Pierce # 34080. To confirm that equal loading and transfer of proteins had occurred, PVDF membranes were subsequently stripped using Restore™ Western Blot Stripping Buffer (Pierce Cat # 21059), blocked in 5% nonfat milk in TTBS for 2 hours and re-probed with rabbit β-actin (abcam # ab8227-50 0.6 mg/ml) at 1:5000 for 3 hours at room temperature, followed by six washes of 5 minutes, incubation in goat anti-rabbit HRP conjugated 1:5000 (Bio-Rad Cat # 172-1013). Then, after the same sequence of washes, bands were visualized with the same chemoluminescent substrate as described before and exposed to Hyperfilm (Amersham Biosciences, Piscataway, NJ) for 5 to 10 seconds.

Protein levels were quantified using a scanner EPSON 1650 and Scion Image for Windows software.

Results: The first experiment involved a set of animals that was not in the current study but included a range of ages to optimize the procedures and establish an age effect. Figure 20 shows that there is a progressive loss of synaptophysin with age that is not due to differences in protein loading across animals. A regression analysis indicates that age was a significant predictor of the level of synaptophysin in the prefrontal cortex ($F(1,8) = 24.67$, $p < 0.002$) and accounted for 77.8% of the variance (adjusted for β-actin levels).

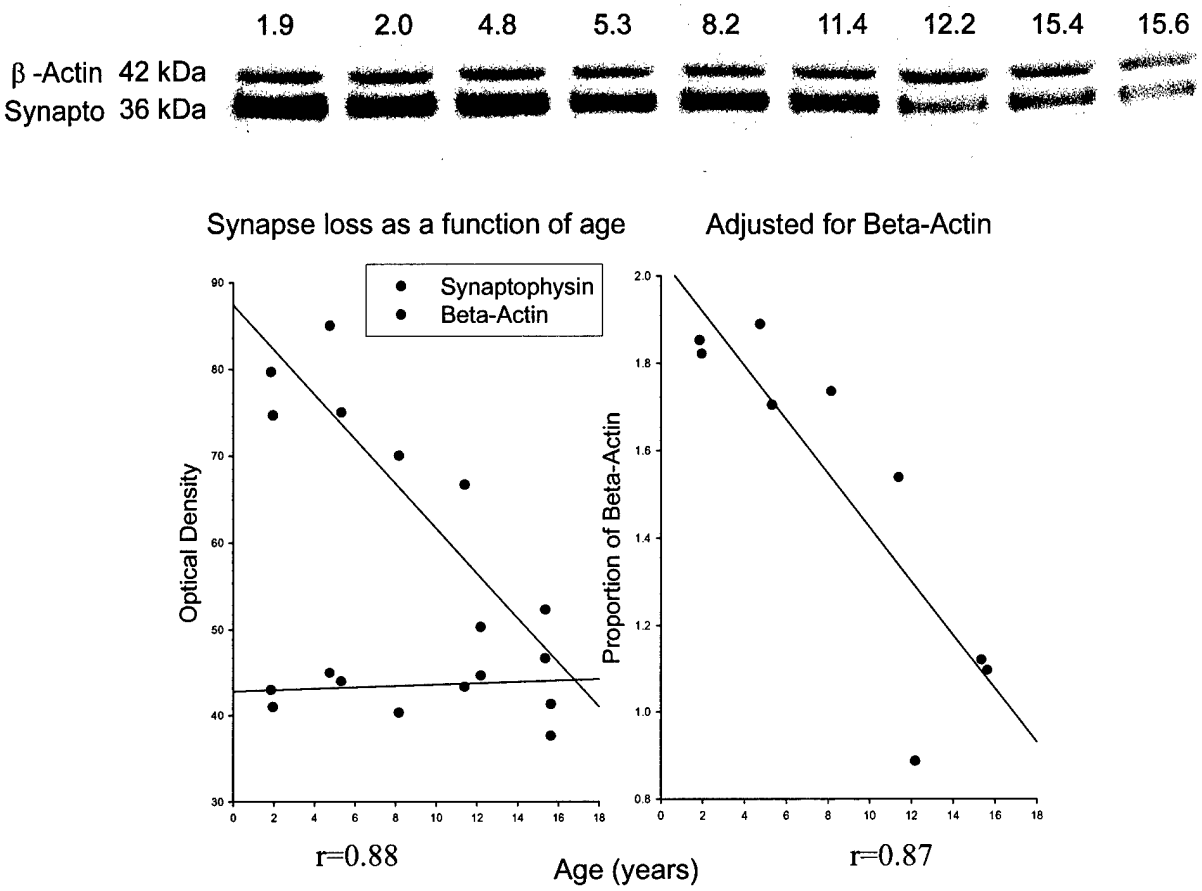

Figure 20. Synaptophysin in the prefrontal cortex decreases as a function of age. Western blots from prefrontal cortex samples were probed for β-actin as a protein loading control and for synaptophysin. β-actin remained relatively constant with age, whereas synaptophysin decreased with age. Plots show individual data points representing the optical density (OD) of the amount of synaptophysin and β-actin confirm quantitatively the qualitative differences observed in the Western blot. In addition, when each samples is corrected for total β-actin, a clear linear decline in synaptophysin is observed with age.

The second set of experiments included 23 aged animals from the longitudinal study and 5 young animals that were added for age comparisons. An ANOVA was used to test for main effects of treatment. Figure 21 shows that in the prefrontal cortex, young animals ($n = 5$) had significantly higher synaptophysin protein levels than old animals ($n = 6$ aged untreated controls) ($t(9) = 2.32$, $p < 0.045$), but there were no overall treatment effects ($F(3,22) = 0.278$, $p < 0.841$). Within each treatment group there was a large amount of individual variability with some animals showing synaptophysin levels in the range of the young animals and others lower. A comparison of animals receiving the diet versus those fed the control diet showed no significant differences ($t(21) = 0.462$, $p < 0.649$), nor did the behavioral enrichment groups differ ($t(21) = 0.805$, $p < 0.430$).

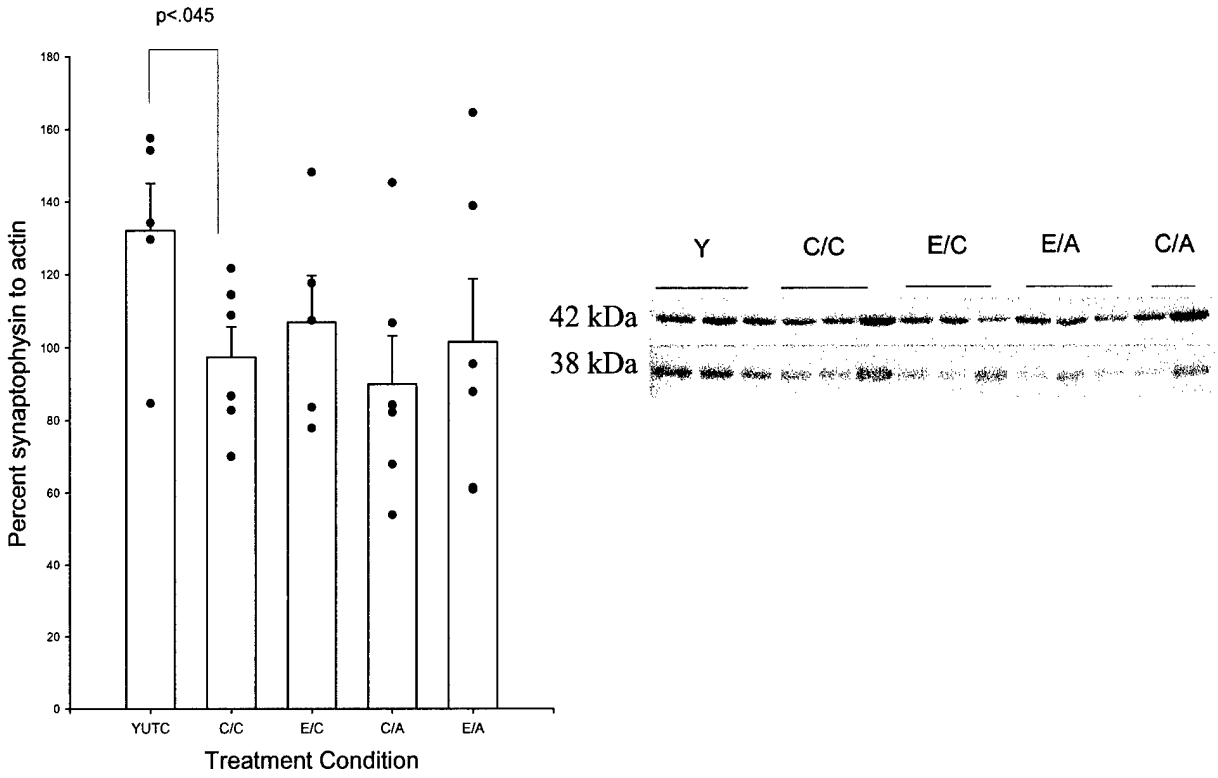

Figure 21. Synaptophysin in the prefrontal cortex is decreased with age but did not vary as a function of treatment condition. Individual optical densities (OD) are plotted as a function of treatment group. Bars represent means, and error bars represent standard errors of the mean. Untreated aged dogs showed significantly lower synaptophysin levels than young dogs. Treatment with behavioral enrichment and/or antioxidant diet had no effect on synaptophysin. A representative Western blot shows a similar age effect but no treatment effect. C/C = control enrichment/control diet, E/C = cognitive enrichment/control diet, C/A = control enrichment/antioxidant diet, E/A = behavioral enrichment/antioxidant diet.

As shown in Figure 22, there was a trend toward young animals having lower levels of synaptophysin in the hippocampus, but these differences did not reach statistical significance ($t(9) = 1.33$, $p < 0.216$). An ANOVA also showed no treatment effect on synaptophysin in the hippocampus ($F(3,22) = 0.77$, $p < 0.525$).

Future projects will include quantitative immunohistochemistry using synaptophysin antibodies as illustrated in Figure 23. Other markers for synapses will be incorporated over the next year of the anatomical studies to confirm and extend these initial findings.

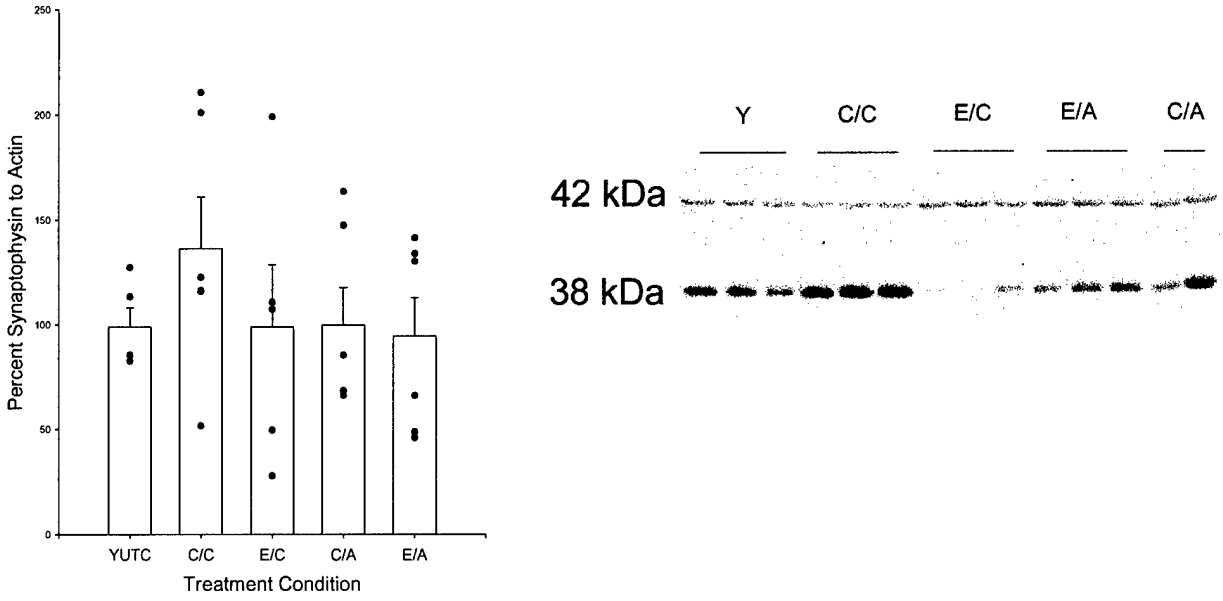

Figure 22. Synaptophysin in the hippocampus did not change as a function of age and treatment. Individual optical densities (OD) are plotted as a function of age and treatment group. Bars represent means, and error bars represent standard errors of the mean. Untreated aged dogs showed higher synaptophysin levels than young dogs, but these differences did not reach statistical significance. Treatment with behavioral enrichment and/or antioxidant diet had no effect on synaptophysin in the hippocampus. A representative Western blot shows a similar age effect but no treatment effect. C/C = control enrichment/control diet, E/C = cognitive enrichment/control diet, C/A = control enrichment/antioxidant diet, E/A = behavioral enrichment/antioxidant diet.

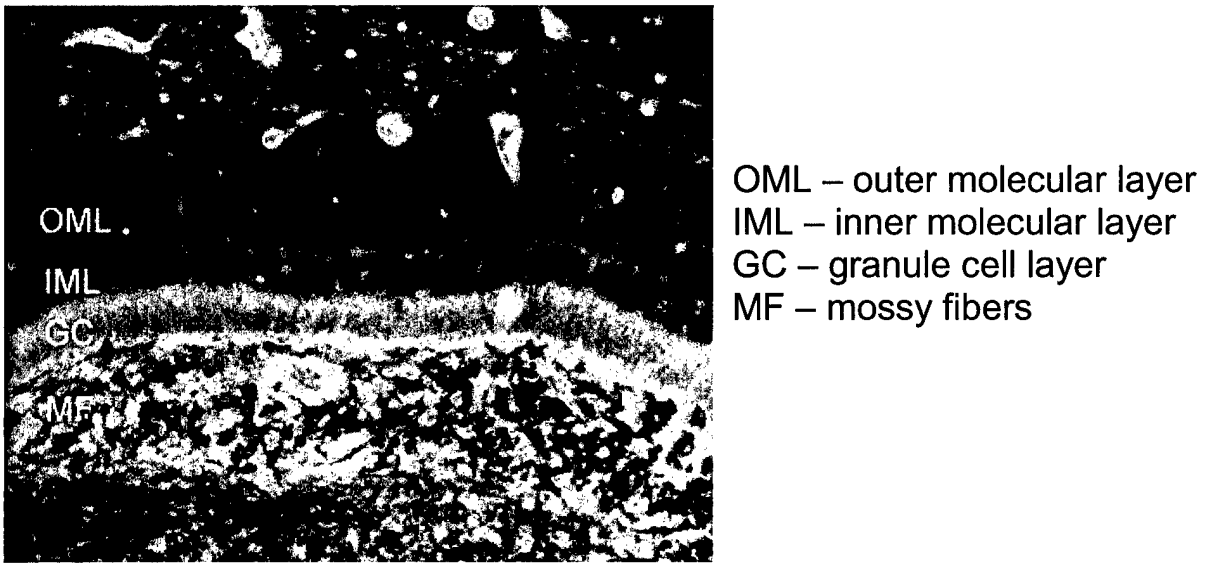

Figure 23. Synaptophysin immunohistochemistry in the hippocampus from an aged animal will be used for further synapse quantification. Synapses and entorhinal cortex neuron terminals can be clearly distinguished in this image and can be used in quantification studies.

Summary: Synaptophysin levels decrease as a function of age in the prefrontal cortex both in a series of archived tissue samples and in the current study. Neither behavioral nor dietary enrichment changed synaptophysin levels in the prefrontal cortex of aged animals. In the hippocampus, there was a trend toward lower synaptophysin levels relative to young animals. Although not statistically significant, there was a trend for synaptophysin to be lower in the treated aged animals that untreated controls to levels that were more comparable to young animals. Using this single marker as initially proposed for the study suggests that the two treatments did not have the expected effects on synaptophysin levels. However, additional markers and methods will now be included to expand and replicate this study. Because synaptophysin is a structural correlate of *number* of synapses in a specific brain region, it may not reflect treatment effects because it is unlikely to be sensitive to changes in synapse *function.*

L. Serum Brain-Derived Neurotrophic Factor as a Function of Age and Treatment Condition

Physical exercise, which was a component of the behavioral enrichment treatment in the current study, is thought to improve brain function by the upregulation of growth factors. In particular, the hippocampus can significantly increase production of one growth factor, brain-derived neurotrophic factor (BDNF) in response to exercise [44, 45]. Thus, one of the endpoint markers we initially proposed for this study was to measure changes in BDNF protein level. Specifically, we hypothesized that dogs receiving the behavioral enrichment would show an increase in BDNF. Since the study was started, a new enzyme-linked immunosorbant assay (ELISA) became commercially available and has allowed us to measure the amount of BDNF in serum samples. The advantage to developing this method was to test whether noninvasive peripheral samples show BDNF changes in response to treatment or to age that may subsequently serve as an outcome measure in clinical trials.

Methods: To measure BDNF, archived serum samples from two time points (baseline and in the last year of the study) were assayed in the longitudinal study dogs. BDNF protein was assessed using the BDNF E-Max ELISA kit (Promega Corporation, Madison, WI) according to manufacturer's recommendations. Efficiency of detection has been confirmed with spike controls, and has been determined to result in greater than 90% efficiency. For protein extraction, dissected brain tissue was homogenized in lysis buffer (18 µl/mg tissue) containing 137 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1% NP40, 10% glycerol, 1 mM PMSF, leupeptin (1 µg/ml), sodium vanadate (0.5 mM), and AEBSF (100 mg/ml). Homogenized samples were

diluted in four volumes of DPBS buffer (0.2 g KCl, 8.0 g NaCl, 0.2 g KH_2PO_4, 1.15 g Na_2HPO_4, 654 µl 1 M $MgCl_2$, 905 µl 1 M $CaCl_2$) before being acidified to less than pH 3.0 with 1 N HCl (15 min, room temperature). 1 N NaOH was then added to all samples to achieve a pH of approximately 7.5. Samples were then centrifuged for 3 minutes (14,000 rpm, 3 minutes, 4°C) and supernatant collected. For the ELISA, 96-well flat-bottomed Immulon-2 plates (DYNEX Technologies, Inc., Chantilly, VA) were incubated overnight at 4°C with carbonate coating buffer containing anti-BDNF monoclonal antibody. Plates were blocked for 1 hour with block and sample (B&S) buffer, followed by incubation with samples and BDNF standards for 2 hours at room temperature with shaking. A standard curve was established using serial dilutions of known amounts of BDNF ranging from 0–500 pg/ml, diluted in B&S buffer. Plates were washed five times with TBST (20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20 in milli-Q water), followed by 2 hours incubation (room temperature) with anti-human BDNF polyclonal antibody, five washes with TBST, and 1 hour incubation (room temperature) with horseradish peroxidase. Enzyme solution (TMB One) was brought to room temperature in advance and subsequently incubated on the plate for 10 minutes (room temperature). After samples turned blue, the reaction was stopped with 1 N HCl and the absorbance read at 450 nm using an automated plate reader.

Results: To measure age effects of BDNF in serum, a total of 35 animals ranging in age from 3.5–15.2 years were included in the study. Hill's provided the young animals. The aged animals were LRRI beagles included in the intervention study. Age was a significant predictor of BDNF in serum, with older animals having higher levels ($r = 0.51$, $p < 0.002$, $n = 35$; Log_{10} BDNF $r = 0.60$, $p < 0.0001$, $n = 35$). A comparison of young and old animals as a group also showed that old animals exhibited higher serum BDNF ($t(33) = 3.12$, $p < 0.004$) as shown in Figure 24.

Within the aged group of dogs ($n = 17$ with BDNF measures at both baseline and in the third year of the study) a repeated-measures ANOVA was used to detect treatment effects. Overall, there was a significant effect of time on serum BNDF ($F(1,13) = 6.69$, $p < 0.023$), and the interaction between time and treatment condition approached significance ($F(3,13) = 2.79$, $p < 0.082$) (Figure 25). A difference score between baseline and Year 3 serum measures was determined by calculating the difference in serum BDNF. A comparison of changes in BDNF across time over the four treatment conditions revealed that the antioxidant-treated dogs showed little change over the 2 years, whereas the control diet animals showed a rise in serum BDNF

($t(15) = 2.99$, $p < 0.009$) (Figure 26). The behavioral enrichment component of the treatment had no effect on serum BDNF.

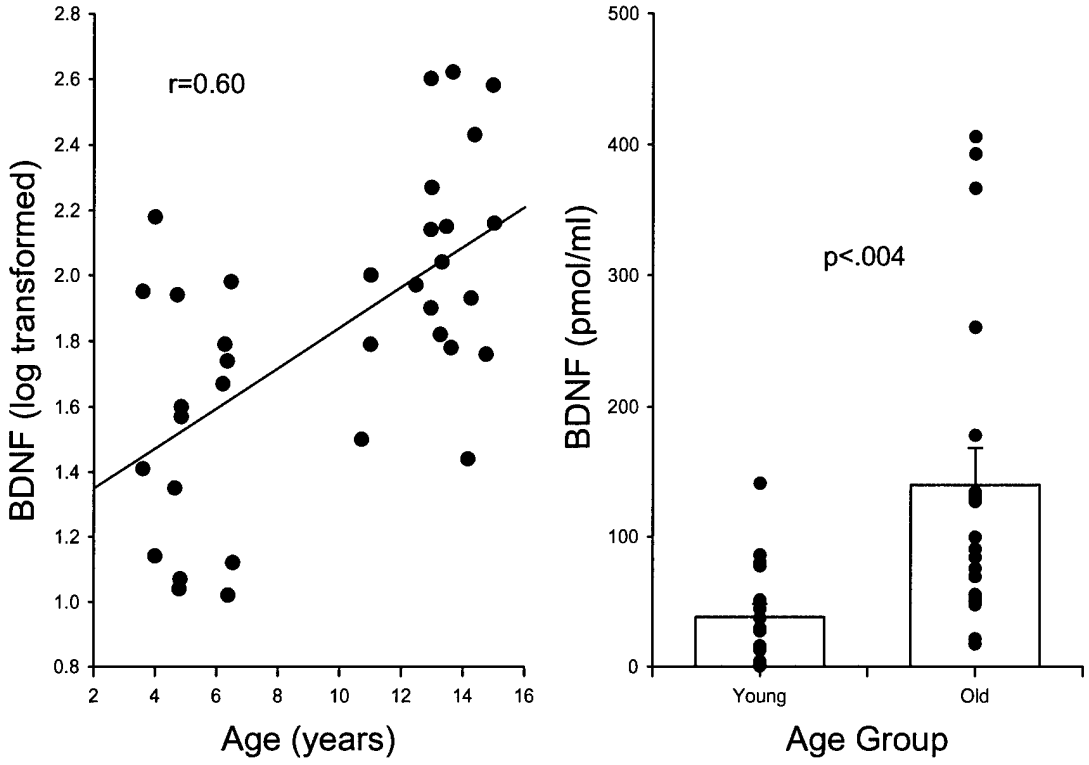

Figure 24. Brain-derived neurotrophic factor (BDNF) increases in serum with age. BDNF was measured using a commercially available ELISA method, and individual data points are plotted as a function of age. The line represents the regression analysis. A second comparison placed animals into either a young or old age group and showed higher individual variability with age, but BDNF was significantly increased in the older group. Bars represent the mean and error bars represent the standard error of the mean.

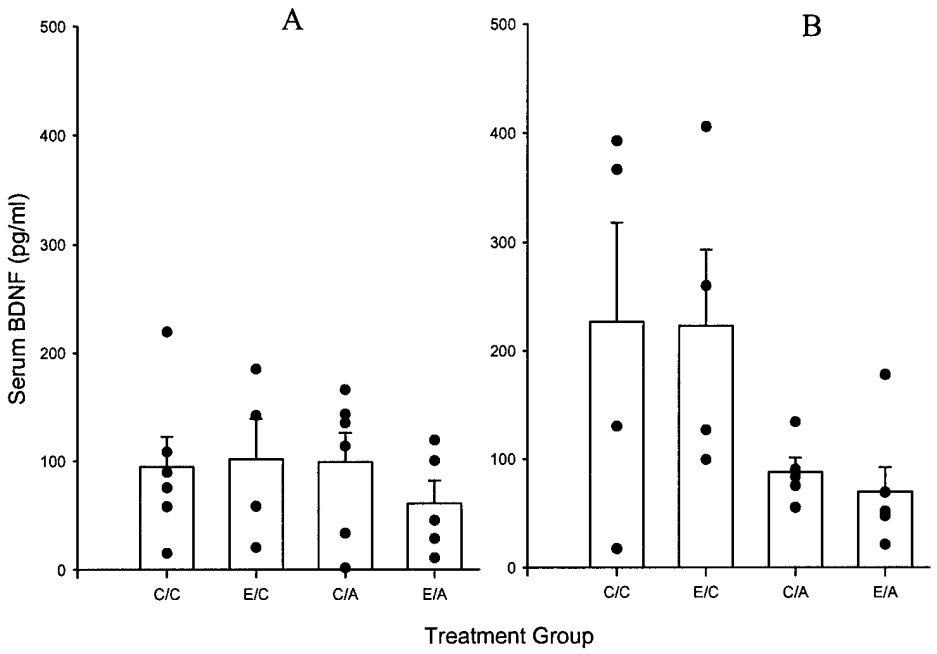

Figure 25. Serum BDNF rises over time and is maintained in treated animals. Serum from two different time points was used to measure rate of change with age and response to treatment. Individual data points are plotted as a function of treatment group for each time point. A. At baseline, no significant treatment effects were observed. B. Serum take after 2.5 years of treatment shows higher BDNF levels in animals receiving the control diet; whereas the antioxidant diet was associated with similar levels to baseline. Bars represent the mean, and error bars represent the standard error of the mean.

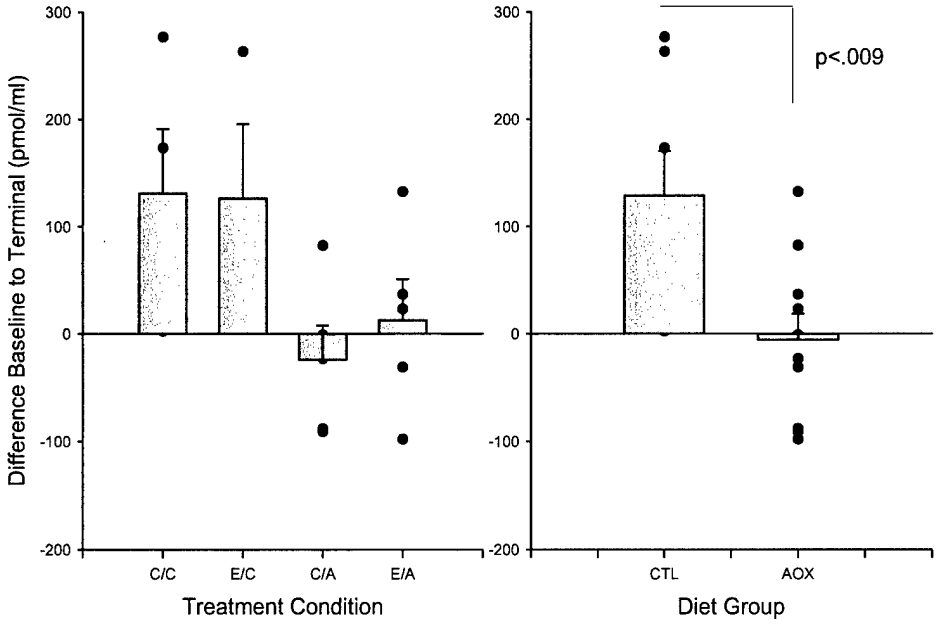

Figure 26. Rate of change in BDNF is slowed in animals fed an antioxidant diet. The average difference in the amount of BDNF at baseline and from the terminal sample is plotted as a function of treatment condition. Note that animals receiving the control diet, regardless of behavioral enrichment, show a progressive increase in serum BDNF. Animals provided with a diet rich in antioxidants show little change over 2.5 years. Bars represent the mean, and error bars represent the standard error of the mean. C/C = control enrichment/control diet, E/C = cognitive enrichment/control diet, C/A = control enrichment/antioxidant diet, E/A = behavioral enrichment/antioxidant diet.

Summary: Serum BDNF rises as a function of age in dogs. An antioxidant-rich diet, but not behavioral enrichment, reduces age-dependent increases in BDNF and maintains these levels over a period of 2 years. We had predicted that BDNF would decrease with age and that this would be reversed with treatment. It is possible that decreased serum BDNF may reflect increased BDNF, with the protein being sequestered and used by neurons in young or treated aged animals. To test this hypothesis, measurements of BDNF will be conducted over the next year. If this proves to be a reasonable hypothesis, then the measurement of serum BDNF may serve as a useful outcome measure in human clinical trials intended to improve brain aging through either diet or exercise.

M. Neurogenesis as a Function of Age and Treatment in the Hippocampus and Frontal Cortex

The current study included a behavioral enrichment component that included physical exercise, environmental enrichment, and cognitive enrichment. Based on the existing literature, we predicted enhanced neurogenesis in animals provided with this treatment.

New neurons are continuously being born in the dentate gyrus of the hippocampus in adult animals. One way to label these new neurons is by providing cells with bromo-deoxyuridine (BrdU), which will be incorporated into newly synthesized DNA. BrdU is visualized using immunocytochemical techniques and an antibody against BrdU. Two phases of neurogenesis can be evaluated using this technique: proliferation and survival. Proliferation is evaluated by using a short time interval between the last injection of BrdU and sacrifice; cells labeled at this time are newly "born." Survival is detected by extending the length of time between the last BrdU injection and detection; cells labeled at this timepoint are those that have survived the time interval [46].

The hippocampus of adult monkeys and humans exhibit neurogenesis in the dentate gyrus using a number of different injection and survival protocols [47–50]. Aged animals show a reduced ability to generate new neurons [51]. The extent of neurogenesis can be modified by interventions such as an enriched environment, exercise, and learning [49, 51–53].

Because our primary interest was in neurogenesis, we selected a time interval of 2 weeks. In previous reports we illustrated that the technical aspects of the study could be conducted successfully using a group of pilot dogs. In the current study, preliminary data will be presented. The full quantification of the extent of neurogenesis is ongoing.

Methods: Two weeks prior to euthanasia, dogs were administered BrdU that will be incorporated into the DNA of new cells in the brain, as per our study protocol. BrdU was diluted in physiological saline and given at a dose of 25 mg/kg. Animals were given a total of five i.v. injections, once per day. A 9-day delay intervened between the last injection and euthanasia. Thus, a total of 14 days between the first injection and euthanasia allowed the incorporation of BrdU into new and surviving cells.

The standard brain preparation protocol described previously (Section J) was used. The left half of the brain has been serially and exhaustively sectioned on all 24 animals (including the four animals that were euthanized or died earlier in the study) for anatomical studies. For age comparisons, five additional young animals that were also used for the measures of mitochondrial function were given BrdU using an identical protocol.

To visualize new cells, 40-m thick free-floating sections were first pretreated with formamide (50%/2xSSC for 2 hours at 65°C next concentration and time) to denature DNA that is wrapped around histones and thus revealing the BrdU epitopes. The tissue was subsequently incubated in anti-BrdU antibody (Novocastra,UK, distributed by Vector Laboratories, Inc., 1:200) overnight at room temperature. After a series of washes, the tissue was incubated in biotinylated anti-mouse, followed by the avidin-biotin complex and visualized with brown DAB and horseradish peroxidase. In order to identify the cell phenotype of BrdU positive nuclei, the tissue was subsequently incubated in either anti-glial acid fibrillary protein (GFAP Dako, Carpenteria, CA, 1:8000 for the first round) to identify astrocytes, or in NeuN (Chemicon, Temecula, CA, 1:1000) to identify neurons, or in anti-HLA-DR (Dako, Carpenteria, CA, 1:100) to identify microglial cells, or in a cocktail of antibodies (myelin CNPase, Sternberger Monoclonal, Inc., Lutherville, MD, 1:400 and myelin basic protein sequence 70-89, 1:10,000) to visualize oligodendroglia.

Results: Figure 27 shows that the injection protocol allowed the incorporation of BrdU into the DNA of replicating cells. Double labels to identify the phenotype of new cells are being optimized. We have now included markers to immunostain oligodendroglia because these are another cell type that undergoes replication. We have obtained additional funds from the National Institute on Aging to conduct stereology-based cell counts for total neuron number, and for total new neurons. These studies are labor intensive and are still ongoing, but we anticipate

obtaining measures of numbers of new astrocytes, microglia, oligodendroglia, and neurons in specific brain regions using a C.A.S.T. system (Olympus Corporation, Melville, NY).

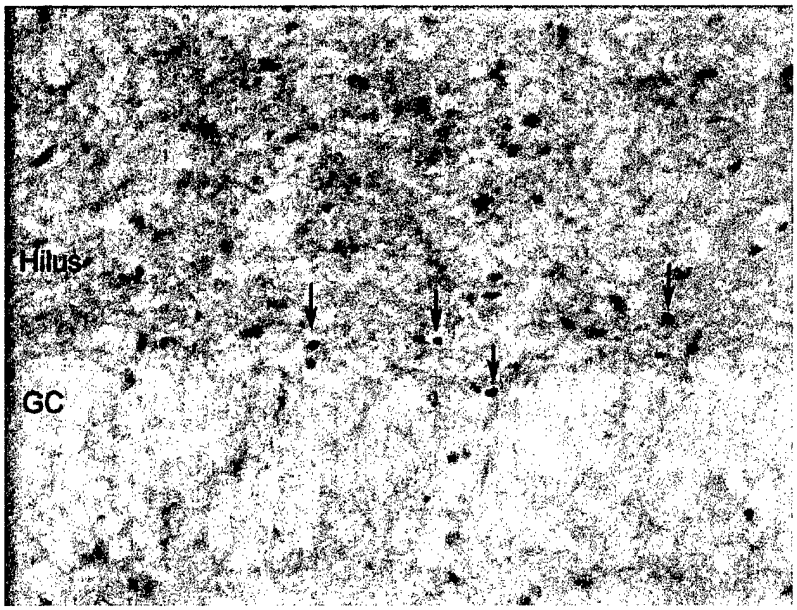

Figure 27. Neurogenesis in the aged canine brain. A photograph of a section from the hippocampus of an aged animal given the compound BrdU, which is incorporated into replicating DNA. Arrows point to nuclei containing BrdU in the hilus of the hippocampus. Sections were double labeled with a cocktail of markers detecting oligodendroglial cells and show that positive neurons are not of this cell phenotype. GC = granule cells.

Summary: We have successfully used BrdU to identify new cells in the brains of animals from the longitudinal study. The methodology for identifying the phenotype is being optimized, and new markers being added as required. In our initial proposal we outlined experiments to measure whether either or both of the treatments would lead to the production or enhanced survival of new neurons. Since this time, unbiased sampling techniques and stereology have become the "gold standard" for obtaining cell counts. To accommodate this development in the field, the left hemisphere from every animal in the study was serially sectioned and will be used to identify new neurons. The study is subsequently more labor-intensive than initially planned, but supplemental funding was obtained from the NIA to conduct this study as precisely as possible. We anticipate that these data will be collected and written over the next year.

III. KEY RESEARCH ACCOMPLISHMENTS

The cognitive testing phase of the study has been completed, and the last set of results is either being written for publication or in submission. A large set of the data has already been published. Currently, the neurobiological studies are the primary focus and will continue well

past the end of the funding period. As needed, supplemental and additional funding has or will be obtained to maintain momentum. We anticipate that the first manuscript describing the effects of the antioxidant diet on the accumulation of Aβ will be in submission by the end of 2003. The following are key research accomplishments from each of the studies reported in this progress report.

- The cognitive testing portion of the longitudinal study has been completed, and the last set of manuscripts are being prepared for submission for publication. The results from the cognitive testing phase of the study provide evidence that behavioral and dietary enrichment can work together in a synergistic fashion to attenuate age-dependent cognitive decline. Both interventions resulted in consistent and long-lasting improvements in cognitive function. The antioxidant supplement dose levels are well within ranges used in human studies and thus can be translated into pilot clinical trials to reduce age-associated and disease-associated cognitive impairments.

- No adverse general health effects were observed by physical examinations, urinalysis, hematology, or clinical chemistry values. The diet did increase serum vitamin E levels, and no significant changes were observed in clotting factors after several years on the diet.

- Longitudinal age-dependent increases in ventricular volume may indicate a general atrophy of cortical structures over time. These age-effects were reduced by providing animals with a diet rich in antioxidants but not by providing behavioral enrichment. Hippocampal volume declined with age, but this age-effect could also be slowed by treating animals with a diet rich in antioxidants. The more dramatic age changes occurred after Year 2 of the study, highlighting the usefulness of longitudinal (as opposed to cross-sectional) imaging studies. MRI provides another noninvasive method to monitor aging and treatment effects in future human clinical trials.

- One of the possible mechanisms underlying the enhanced cognitive function observed in aged dogs treated with an antioxidant diet is by improved mitochondrial function. Reactive oxygen species (ROS), the primary cause of oxidative damage to proteins, lipids, and DNA/RNA within cells, is significantly higher in aged animals relative to young controls. ROS production was reduced in aged animals provided with a diet rich in antioxidants and mitochondrial co-factors. Complex I, the first component of the

mitochondrial complex chain, exhibits significantly higher activity in young animals compared to old animals. The activity of this enzyme was improved in aged animals provided with a diet rich in a broad spectrum of cellular antioxidants as well as mitochondrial co-factors.

- Cellular oxidative damage measured by the accumulation of protein oxidation products (carbonyls, enzyme dysfunction) and lipid peroxidation (malondialdehyde) in the brain did not change in response to either of the treatment conditions. Age effects were not observed in any of these endpoint measures except for higher GS activity in the temporal cortex of young animals. These results may suggest that the antioxidant diet had little effect on cellular oxidative damage, and changes in lipid peroxidation and protein oxidation do not account for cognitive improvements.

- A new measure of lipid peroxidation, isoprostane, was introduced into the study to confirm the results of the study on malondialdehyde accumulation. Young animals showed lower isoprostane levels in plasma and in the temporal cortex than old animals. No difference in temporal cortex accumulation of isoprostane was observed in response to the antioxidant diet. The inclusion of plasma measures of isoprostane may still serve as a useful outcome measure in human clinical trials due to its age-dependency; but, the combination of components used in the canine study may have had little impact on lipid peroxidation in the brain.

- As proposed initially, Aβ deposition was quantified in aged animals in the longitudinal study. Aβ deposition in the antioxidant treated animals was lower in the parietal and entorhinal but not occipital nor prefrontal cortex. In addition, the antioxidant diet considerably reduced individual variability, indicating that all old dogs benefited from the treatment. Aβ reduction was selective for the antioxidant treatment because no Aβ-lowering effects were observed in animals receiving the enriched environment protocol alone. These results suggest that a diet rich in antioxidants can slow but not reverse Aβ deposition, and we are currently studying the possible mechanisms underlying this effect. The dose levels used in the current study are consistent with human clinical trials and indicate that dietary supplementation with a broad spectrum of antioxidants may prove beneficial for human brain aging.

- We report the first age study of synaptophysin in the canine model and show that synaptophysin declines with age in the prefrontal cortex but may actually rise with age in the hippocampus. The treatment showed mild effects on synaptophysin levels but none reached statistical significance. Thus, the mechanism underlying cognitive improvements in animals provided with behavioral enrichment will be expanded to include other markers of synaptic growth in future studies.

- Another potential marker of neuron growth or health is BNDF. Serum BDNF rises as a function of age in dogs. A diet rich in antioxidants, but not behavioral enrichment, reduces age-dependent increases in BDNF and maintains these levels over a period of 2 years. This is the first serum marker that we have identified that increased with age over time during the study and was maintained at lower levels comparable to younger animals in response to treatment. The next series of studies will examine BDNF levels in the brain.

- We have successfully used BrdU to identify new cells in the brains of animals from the longitudinal study. The methodology for identifying the phenotype is being optimized, and new markers are being added as required. Unbaised sampling and stereology will be used to obtain reliable and accurate counts of the number of new and surviving neurons in the study animals. We anticipate that these data will be collected and written over the next year.

- To summarize the series of endpoint markers described in this final report and the effects of age and treatment, the reader is referred to Table 6. Several of the biological endpoint measures show changes in response to treatment condition. In particular, brain atrophy was slowed, hippocampal volume was maintained, mitochondrial function was improved, Aβ accumulation was decreased, and serum BDNF was maintained all as a consequence of the treatment protocols. All or some combination of these outcome measures reflecting possible neurobiological mechanism underlying improved neuron function may account for the observed cognitive improvements. Additional studies will continue over the next year, and we anticipate making significant progress.

Marker	Age Effects	Treatment Effects	Antioxidant Diet Effect	Behavioral Enrichment Effect
Plasma Vitamin E	Increased with age	Increased with treatment	Increased	No change
Ventricle Volume	Increased with age	Decreased with treatment	Decreased	No change
Hippocampal Volume	Decreased with age	Maintained with treatment	Maintained	No change
Mitochondrial ROS	Increased with age	Decreased with treatment	Decreased	No change
Mitochondrial protein oxidation	Increased with age	Increased with treatment	Increased	No change
Mitochondrial Complex I	Decreased with age	Increased with treatment	Increased	No change
Plasma IsoP	Decreased with age	Decreased with treatment	Decreased	No change
CSF IsoP	Decreased with age	Increased with treatment	Increased	No change
Brain IsoP	Increased with age	No change	No change	No change
Brain MDA	No change	No change	No change	No change
Brain Carbonyls	No change	No change	No change	No change
Glutamine Synthetase	Decreased with age	No change	No change	No change
Beta-Amyloid	Increased with age	Decreased with treatment	Decreased	No change
Hippocampal Synaptophysin	Increased with age	No change	No change	No change
Prefrontal Synaptophysin	Decreased with age			
Serum BDNF	Increased with age	Decreased with treatment	Decreased	No change
Serum BDNF Change over time	Increased with age	No change	No change (maintained)	No change

IV. REPORTABLE OUTCOMES

Over the entire funding period we have published 8 papers and presented 12 abstracts. Over the last year of the study, 2 papers were published, 1 is in press, and 2 abstracts were presented. These publications from this last year are indicated in the list below in all bold lettering, and those available at the time of this report are provided in Appendix A.

Abstracts and Conferences:

1. Head, E., Liu, J., Ames, B.N., Muggenburg, B.A., Milgram, N.W., and Cotman, C.W. Oxidative stress, β-amyloid and cognitive function in the aged canine brain. Society for Neuroscience Abstracts, **2000**.

2. Ikeda-Douglas, C., Callahan, H., Head, E., Cotman, C., Araujo, J., Chan, A., Estrada, J., Muggenberg, B., Zicker, S., and Milgram, N.W. Visual object recognition in beagle

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3. Milgram, N.W., Estrada, J., Ikeda-Douglas, C., Drosz, J., Castillo, J., Magre, O., Head, E., Cotman, C., Murphey, H., Holowachuk, D., Muggenberg, B., and Zicker, S. Landmark discrimination learning in the dog: Effect of diet, age, and test sophistication. Society for Neuroscience Abstracts, **2000**.

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9. Head, E., Liu, J., Muggenburg, B.A., Murphey, H., Ikeda-Douglas, C., Zicker, S., Ames, B.N., Milgram, N.W., and Cotman, C.W. A longitudinal dietary antioxidant intervention

in aged canines improves learning reduces peripheral measures of oxidative damage. Society for Neuroscience, **2002**.

10. Ikeda-Douglas, C.J., Murphey, H., Muggenburg, B., Head, E., Cotman, C.W., Zicker, S.C., and Milgram, N.W. Long-term maintenance of an antioxidant enriched food plus behavioral enrichment markedly delays age related cognitive decline in beagle dogs. Society for Neuroscience, **2002**.

11. Tapp, P.D., Siwak, C.T., Zicker, S.C., Head, E., Muggenburg, B.A., Cotman, C.W., Murphey, H.L., Ikeda-Douglas, C.J., and Milgram, N.W. An antioxidant enriched diet improves concept learning in aged dogs. Society for Neuroscience, 2003 (not yet available).

12. Pop, V., Head, D., Nistor, M., Milgram, N.W., Muggenburg, B.A., and Cotman, C.W. Reduced Aβ deposition with long-term antioxidant diet treatment in aged canines. Society for Neuroscience, 2003 (not yet available).

Manuscripts In Press or Published:

1. Milgram, N.W., Zicker, S.C., Head, E., Muggenburg, B.A., Murphey,H., Ikeda-Douglas, C., and Cotman, C.W. (2002). Dietary enrichment counteracts age-associated cognitive dysfunction in canines. Neurobiology of Aging, 23: 737-745.

2. Head, E., Liu, J., Hagen, T.M., Muggenburg, B.A., Milgram, N.W., Ames, B.N., and Cotman, C.W. (2002). Oxidative damage increases with age in a canine model of human brain aging. Journal of Neurochemistry, 82: 375-381.

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learning in the dog: Effects of age, an antioxidant fortified diet, and cognitive strategy. Neuroscience and Biobehavioral Reviews, 26: 679-695.

6. **Siwak, C.T., Tapp, P.D., Zicker, S.C., Murphey, H.L., Muggenburg, B.A., Head, E., Cotman, C.W., and Milgram, N.W. (2003). Locomotor activity rhythms in dogs vary with age and cognitive status. Behavioral Neuroscience, 117(4): 813-824.**

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9. **Milgram, N.W., Head, E., Vicker, S.C., Ikeda-Douglas, C.J., Murphey, H., Muggenburg, B.A., Siwak, C., Tapp, D., and Cotman, C.W. (in submission). Learning ability in aged beagle dogs is preserved by behavioral environment and dietary fortification: A 2-year longitudinal study. Neurobiology of Aging.**

V. CONCLUSIONS

The goals for Year 5 were to continue the neurobiological studies in animals that completed the cognitive portion of the longitudinal experiments. The results of the study indicate that both treatments, the behavioral enrichment and the antioxidant diet, significantly impact cognitive aging. The results from the combination treatment group suggest that the two interventions are additive. The study of nontreated aged animals has led to the first longitudinal study of aging reported in a higher mammalian species and shows progressive declines in several cognitive domains, particularly those that rely upon the prefrontal cortex. Age-dependent decline can be prevented through the interventions used in the current study with a combination of treatments resulting in a preservation of cognitive function.

In vivo imaging data in the longitudinal study are now providing evidence that the hippocampus may indeed progressively decrease in size in untreated animals and that the interventions may slow the rate of atrophy. In addition, we now have stronger evidence that

ventricular volume rises with age but this effect did not become apparent until after the second year of the study.

The neurobiological studies have been the focus over the last year, and these studies will continue for an additional time period. Additional funding was obtained to support a full stereology-based study of cell counts and quantification of neurogenesis. These studies will be completed over the next year.

One of the next phases of the study is also to bring together the cognitive and neurobiological data to determine what mechanisms are responsible for the observed treatment effects. To provide assistance with this phase of the data analysis, a biostatistician will be consulted.

Although the funding for this project from the DAMD is now complete, we will continue our neurobiological studies and the submission and publication of data for the next several years. As necessary, additional markers will be introduced and new collaborations formed. When required, funding will be requested from several sources to support new experiments using tissue from the animals from this study.

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Appendix A

New Reprints and Preprints Resulting from Contract DAMD17-98-1-8622

Behavioral Neuroscience
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Visuospatial Impairments in Aged Canines (*Canis familiaris*): The Role of Cognitive–Behavioral Flexibility

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This study used a novel delayed nonmatching-to-position task to compare visuospatial learning and memory in young and aged beagle dogs (*Canis familiaris*). The task used 3, rather than 2, spatial locations, which markedly increased difficulty. There were striking age differences in acquisition. Most of the aged canines did not learn the task, and those that did showed impaired learning when compared with the young canines. The aged canines also showed reduced maximal working memory capacity compared with the young canines. Analysis of the response patterns of individual canines indicated that the deficits were related to the use of ineffective strategies and inflexibility in strategy modification.

Spatial learning and memory ability is impaired in aged humans (Barnes, 1988; Olton, 1988; Rutledge, Hancock, & LaJuana, 1997; Sharps & Gollin, 1987; Uttl & Graf, 1993; Weber, Brown, & Weldon, 1978) and is a prominent feature of age-related neurobiological disorders including Alzheimer's and Parkinson's diseases (Freedman & Oscar-Berman, 1989). Visuospatial function is also age-sensitive in nonhuman primates (Bachevalier et al., 1991; Bartus, Fleming, & Johnson, 1978; Moss, 1993; Rapp & Amaral, 1991) and rodents (Barnes, 1979; Barnes, Nadel, & Honig, 1980; Colombo & Gallagher, 1998; Dunnett, Martel, & Iversen, 1990; Frick, Baxter, Markowska, Olton, & Price, 1995; Gage, Dunnet, & Bjorklund, 1989; Dunnett, Evenden, & Iversen, 1988; Gallagher & Burwell, 1989; Gallagher, Burwell, & Burchinal, 1993; Gallagher & Pelleymounter, 1988; Rapp, Rosenberg, & Gallagher 1987). Aged primates are deficient in learning delayed response tasks compared with younger primates (Moss, 1993; Rapp & Amaral, 1989) and are also deficient in remembering spatial information when delays are increased (Bachevalier et al., 1991; Bartus et al., 1978; Marriott & Abelson, 1980; Medin, 1969; Rapp & Amaral, 1989; Voytko, 1993). These age-related deficits are not necessarily indicative of global cognitive dysfunction, as visuospatial learning and memory are impaired at an earlier age than object recognition memory (Bachevalier, 1993; Bachevalier et al., 1991; Herndon, Moss, Rosene, & Killiany, 1997; Rapp & Amaral, 1989; Rapp, Kansky, & Roberts, 1997).

We previously described age-dependent deficits in visuospatial function in a canine model of human aging using a delayed nonmatching-to-position (DNMP) task (Adams, Chan, Callahan, & Milgram, 2000; Adams, Chan, Callahan, Siwak, et al., 2000; Head et al., 1995). In this task, dogs are presented with a sample stimulus at one of two spatial locations. After a delay, the sample and an identical stimulus are placed at both spatial locations. To obtain reward, the dog must respond to the location that was not presented in the sample phase. Aged dogs show slower learning and impaired performance at long delays when compared with young dogs. Using these data, we have been able to characterize cognition in different subsets of dogs as reflecting successful, impaired, or severely impaired agers (Adams, Chan, Callahan, & Milgram, 2000; Adams, Chan, Callahan, Siwak, et al., 2000; Head et al., 1995; Milgram, Head, Weiner, & Thomas, 1994).

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The DNMP task can be solved using either of two strategies: remembering where the sample was and using the general rule of avoiding responding to the sample or remembering which direction to respond to, which can be determined from the sample location. Because the location of the correct response can be deduced from the location of the sample, this task can be solved by a non-mnemonic strategy, such as maintaining a fixed posture and orienting toward the correct location over the delay period. Orienting strategies have been observed in rodents performing a similar DNMP task (Chudasama & Muir, 1997). As possible evidence of dogs learning to use orientation strategies to solve this task, we have found that performance of both young and aged dogs can improve markedly with extensive repeated testing, and that dogs of all ages become adept at very long delays. To decrease the likelihood of the subjects using a non-mnemonic solution to solve this visuospatial task, we have developed a new version of the DNMP task, the three-position DNMP (3-DNMP) task.

The modified 3-DNMP task uses three, rather than two, spatial positions. The addition of a third location makes it impossible for the subject to know the correct location before being presented with the test stimuli. Successful performance requires that the subjects remember the location of the sample and learn the general rule of avoiding responding to the sample location in the comparison phase. The present study compares both acquisition and performance at progressively increasing delays on this novel task in a group of young and old dogs.

Method

Subjects

Two groups of beagle dogs (*Canis familiaris*) served as subjects. The first group consisted of 17 young dogs (3–5 years old, 8 males and 9 females). The second group consisted of 48 aged dogs (9–12 years old, 24 males and 24 females). The justification for using the age of 9 as a cutoff was based partly on survival data indicating that 9 years is the point at which mortality begins to accelerate in beagle dogs at the test facility (Muggenburg, Hahn, & Benjamin, 2001). However, almost 90% of the population is still alive. Thus, the dogs that will become successful, impaired, and severely impaired agers are still in the population. In addition, 9 is approximately the age of appearance of β-amyloid protein in the beagle brain (Head, McCleary, Hahn, Milgram & Cotman, 2000). Both groups of dogs came from colonies at the Lovelace Respiratory Research Institute in Albuquerque, New Mexico (8 young and 24 old), and Hill's Pet Food in Topeka, Kansas (9 young and 24 old). All dogs were fed approximately 300 g of dry dog food once daily and were given periodic clinical examinations over the course of the study to ensure that cognitive performance was not affected by deficits in physical, sensory, or motor functioning.

The aged dogs were housed, either singly or in pairs, in pens with continual access to fresh water at the Lovelace Respiratory Research Institute. The young dogs were housed at the animal facility at the University of Toronto at Scarborough, 2 to 4 per room. In all other respects, the dogs were treated identically. In conducting the research, we adhered to the *Guide for the Care and Use of Laboratory Animals*, prepared by the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (National Institutes of Health, 1986).

Testing Apparatus

The test apparatus was a wooden chamber (1.15 m long × 0.609 m wide × 1.08 m high) based on a canine adaptation of the Wisconsin General Test Apparatus (see Figure 1; for a detailed description, see Milgram et al., 1994). The testing chamber was equipped with a sliding Plexiglas food tray with three food wells. Adjustable vertical stainless steel bars at the front of the box provided openings for the dog to obtain food from the food wells. The height of the bars was uniquely set for each dog. The experimenter was separated visually from the dog by a one-way mirror; a hinged wooden door was located below the mirror. Testing was conducted in darkness, except for a 60-W lightbulb attached to the front of the box. Each test trial commenced with the hinged door being opened for

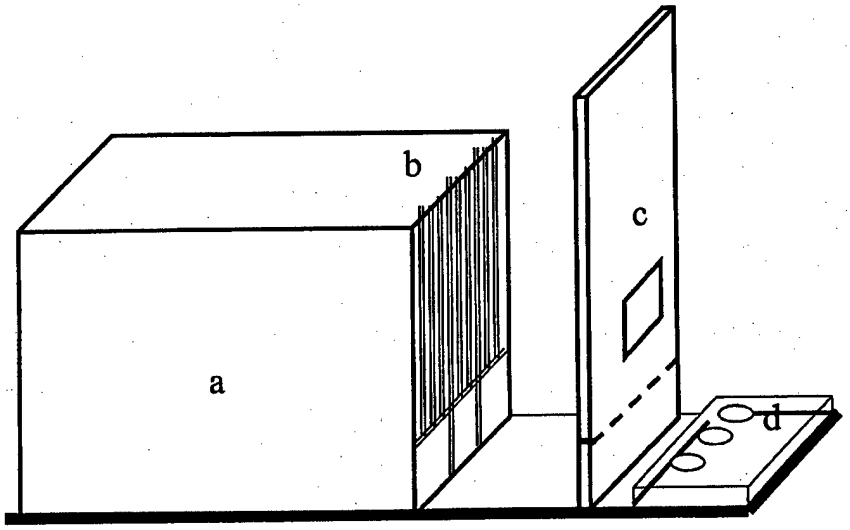

Figure 1. Apparatus used in cognitive testing with dogs. The apparatus contains a rectangular box where the dog resides (a), stainless steel bars of adjustable height that provide three openings (b), a screen with a one-way mirror separating the experimenter and dog (c), and a black Plexiglas presentation tray with three food wells, two lateral and one medial (d).

the presentation of the tray. Approximately 1 cm^3 of Hill's Pet Food pediatric diet was used as the food reward.

Behavioral Testing Protocols

Cognitive testing was conducted in the morning and early afternoon. Before beginning this study, every dog was administered a standard pretraining protocol that was intended to familiarize them with the testing apparatus and procedures (Milgram et al., 1994). The protocol included training on reward and object approach learning, object discrimination learning, and discrimination reversal learning. Half of the dogs in both groups were trained on the 3-DNMP test after completing this pretraining protocol. The other half was first trained on an object recognition task (Callahan, Ikeda-Douglas, Head, Cotman, & Milgram, 2000; Milgram et al., 1994).

In the 3-DNMP task, each trial began with a sample phase, in which a small red block covering a food well was presented at one of three positions: left, center, or right. After the dog displaced the block and obtained the reward, the tray was withdrawn. After a delay, the test phase started with the presentation of both the sample and the nonmatch (an identical red block in one of the two other locations). The dog then had to displace the nonmatch to obtain reward. If the dog responded to the sample (incorrect response), the tray was immediately withdrawn and an error was recorded. To prevent the dogs from using olfactory cues to solve the task, a quantity of food approximately equal to that associated with the nonmatch was stuck to the bottom of the incorrect stimulus. After a 60-s intertrial interval, the sample phase of the next trial was initiated. The memory demands of this task were manipulated by varying the length of delay between the sample and test phase.

Data were manually collected by means of a customized computer program that controlled timing, randomization procedures, and location of sample and nonmatch position, as well as recorded choice-reaction times. Before the start of each trial, the computer emitted a tone that served as a cue for the subject and instructed the experimenter to deliver the food tray. Each trial was initiated when the experimenter pressed a key and simultaneously presented the tray. Each response was recorded by a keypress, which also indicated the end of the trial and signaled the beginning of the intertrial interval.

During the actual training, each dog received 12 trials per day. We used a correction procedure in which the subject was allowed to correct its response only after making its first error on each session. The dogs were initially trained at a 10-s delay until they either completed 600 trials (50 sessions) or passed a two-stage criterion. The first stage involved correctly responding on 11/12 trials or better on 1 day; on 10/12 trials or better over 2 consecutive days; or on 10/12, 9/12, and 10/12 trials over 3 days. To successfully complete the second stage of criterion, they had to respond correctly on at least 70% of the next 36 completed trials (over 3 consecutive days). Thus, a minimum of 4 test days was required to achieve the two-stage criterion. The subjects that passed the criterion at the 10-s delay were then tested at progressively longer delays over 40 sessions. The successive delays used were 20 s, 30 s, 50 s, 70 s, 110 s, and 150 s. To advance to a higher delay, the dogs were required to pass the two-stage criterion at the present delay. The last delay at which a dog was successfully able to pass criterion was considered that dog's maximal memory capacity, that is, the longest interval during which the dog was able to accurately retain spatial information.

Strategies Analysis

Dogs frequently develop a preference for responding to one location when presented with a new task, which we defined as a *positional response bias* (Milgram et al., 1994). A pure positional bias was not possible in this task because of the use of three positions. However, we noted that some dogs showed either a position preference (i.e., responded to a particular position whenever possible) or a position avoidance (i.e., never responded to a particular position when given the opportunity). To quantify this type of positional strategy, we developed the following position bias index (PBI): PBI = |4 − (right-side choices)| + |4 − (center-choices)| + |4 − (left-side choices)|.

Each spatial location was an optional response a total of eight times per session (12 trials) but was the correct location on only four of these. Thus, the range of possible scores on this index varied from 0 (four responses to each of the three locations) to eight (complete avoidance of a particular spatial location).

We also noted that task difficulty varied as a function of the position of the sample. When the sample was presented to either the far left or far right, the correct response was always to the opposite side. When the sample was presented in the center, however, the correct response was to the left of the sample half of the time, and to the right the other half. We characterized these alternatives in terms of three separate problems or subtests, based on the sample–nonmatch configurations (see Figure 2). In the first, the center–nonoption subtest, the sample was presented to either the left or right position and the other lateral well was used as the nonmatch. This configuration is identical to that used in the two-choice DNMP task. The second, the center–correct subtest, involved presenting the sample in one of the two lateral wells and the nonmatch in the center well. The third, the center–incorrect subtest, comprised all trials in which the sample was presented in the center food well.

We distinguished between these subtests because of the possibility that the dogs learned only some of the subtests. For example, when the sample was presented to the dog's right, the dog was always rewarded if it responded toward the opposite side (center or left) on the comparison trial. However, this strategy could not be used to solve the center–incorrect subtest.

On two of the subtests (center–nonoption and center–correct), the location of the correct response was indicated by the location of the sample. The dogs could solve both of these by learning to orient away from the sample. Because this solution could be acquired using associative learning, we use the term *stimulus–response* (S-R) strategy to describe it, on the basis of terminology used by Toates (1998). In contrast, we use the term *cognitive strategy* to describe a solution in which knowledge of the correct response was linked to use of a general rule rather than a particular stimulus (Toates, 1998).

From this perspective, S-R strategies could be used to solve both the center–nonoption and center–correct subtests, as the correct response was to one side of the sample. For example, if the sample appeared at the dog's left, the correct response was toward the dog's right, to either the center or right position. A cognitive strategy could also be used to solve these two subtests but had to be used to solve the center–incorrect subtest. In the center–incorrect subtest, the nonmatch could have appeared in either the left or right position. Thus, the correct response could not be anticipated from the sample position, and a general nonmatching rule had to be used to guide choice response.

To determine the type of strategy used to solve the 3-DNMP task, we calculated percentage accuracy scores for each subtest over the final five sessions of acquisition training and compared the results against random chance performance. Chance performance was determined as a binomial probability function with a 50% probability of selecting one of the two locations in the comparison phase. A dog was classified as using an S-R strategy if it performed better than chance on the center–nonoption and center–correct subtest but no better than chance on the center–incorrect subtest. A dog was classified as using a cognitive strategy if it performed better than chance on all three subtests. A dog was classified as using a position bias if any other subtest performance pattern was present. The specific pattern would be a function of the particular bias the dog possessed.

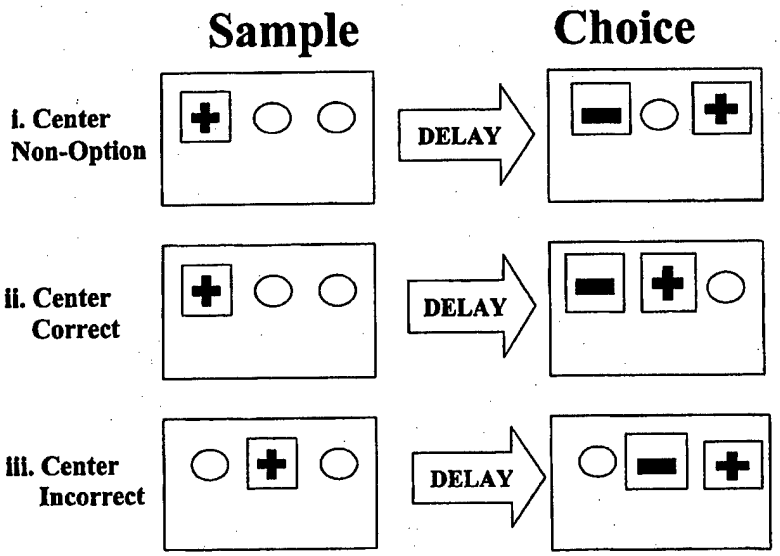

Figure 2. Three-position delayed nonmatching-to-position paradigm and the three associated subtests. Note that two configurations (sample and choice) exist for each of the subtests.

Statistical Analysis

All statistical analyses were conducted with SPSS for Windows 10.0.7 (2000) software package, with an alpha level of 5% ($\alpha = .05$). All univariate repeated measures are Greenhouse–Geiser epsilon corrected. Post hoc Tukey's honestly significant difference (HSD) tests were used for all pairwise comparisons. We used the Fisher exact probability test (2×2 tables) or chi-square test for all comparisons based on category frequencies (nominal data). Finally, we used the Mann–Whitney U test for comparisons of maximal memory, because of the unequal magnitudes between delays (ordinal data).

Results

3-DNMP Acquisition

We compared the groups by using a three-way repeated measures analysis of variance (ANOVA) based on the number of errors committed during training at the 10-s delay. Age (young or old) and sex (male or female) were between-subject variables, and subtest (center–nonoption, center–correct, and center–incorrect) was a within-subject variable. There were significant main effects of age, $F(1, 61) = 61.99$, $p < .01$, and subtest, $F(1.37, 83.55) = 68.25$, $p < .01$, but no effect of sex.

Figure 3 illustrates that the age effect was due to poorer performance by the old dogs. In fact, only 8 of 48 aged dogs passed the two-phase criterion at the 10-s delay. In contrast, 15 of 17 of the young dogs learned the task. A Fisher's exact test indicated that this difference was highly significant ($p < .01$).

We also found a significant Age × Subtest interaction, $F(1.37, 83.55) = 22.10$, $p < .01$ (see Figure 4a). Separate post hoc one-way ANOVAs were conducted on the young and old groups. For the aged dogs, the post hoc comparisons revealed significant differences between all three subtests ($p < .01$), with the most errors committed on the center–incorrect subtest and the least on the center–correct subtest. For the young dogs, post hoc comparisons revealed significantly more errors on the center–incorrect subtest than on the center–nonoption ($p < .02$) and center–correct ($p < .03$) subtests.

Figure 4b also shows the results of analysis restricted to only those dogs that successfully passed the initial criterion ($n = 15$ young, $n = 8$ aged). A three-way repeated measures ANOVA was conducted on the number of errors committed by dogs that passed the preset criterion at a 10-s delay, with age and sex as between-subjects variables and subtest as a within-subject variable. There was a main effect of age, $F(1, 21) = 7.27$, $p < .02$, with aged dogs committing more errors during acquisition than young dogs. There was also a main effect of subtest, $F(1.76, 33.44) = 23.84$, $p < .01$, and a significant Age × Subtest interaction, $F(1.76, 33.44) = 8.28$, $p < .01$. Separate post hoc one-way ANOVAs were conducted on the young and old groups. For the aged dogs, post hoc comparisons

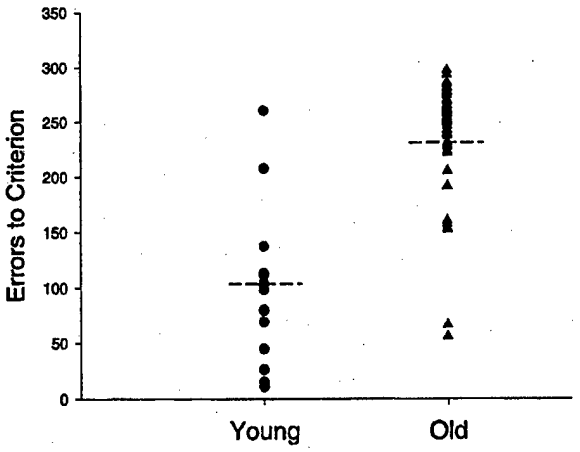

Figure 3. Errors to criterion by individual subjects during the acquisition phase of the three-position delayed nonmatching-to-position task for the young and old groups. The dashed lines show the group averages.

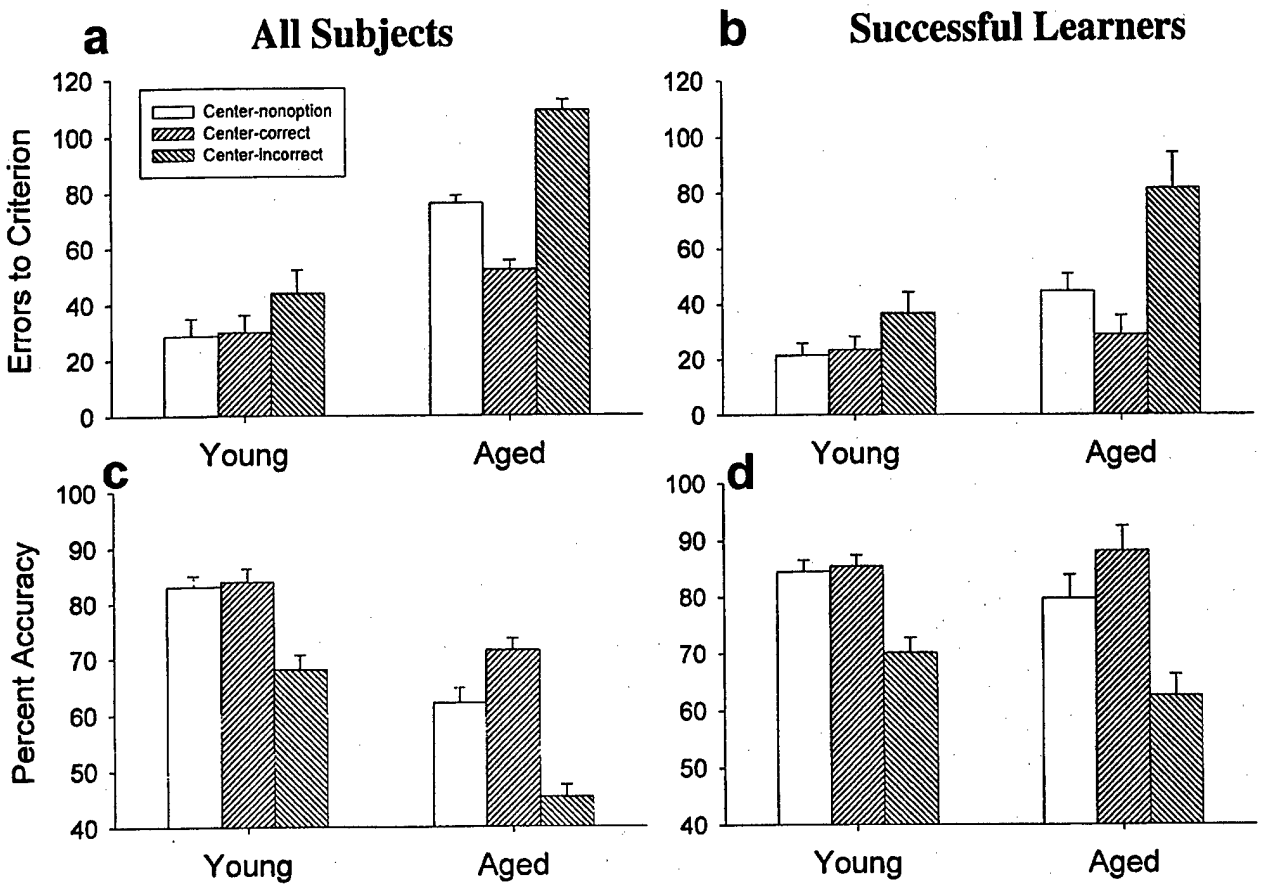

Figure 4. Acquisition errors on the three-position delayed nonmatching-to-position task as a function of age, subtest, and success in learning. The top panels show total errors to criterion during acquisition at the 10-s delay for all subjects (a) and for those that learned the task (b). Note that the aged dogs made more errors than the young dogs and disproportionately more on the center–incorrect subtest. The bottom panels show accuracy scores over the last five training sessions for all subjects (c) and for those that learned the task (d). Error bars represent standard error.

revealed significant differences among all the three subtests ($p <$ .01), with the most errors committed on the center–incorrect subtest and the least on the center–correct subtest. For the young dogs, post hoc comparisons revealed significantly more errors on the center–incorrect subtest than the center–nonoption ($p < .02$) and center–correct ($p < .04$) subtests, which did not significantly differ.

To further examine whether the dogs had not acquired, partially acquired, or completely acquired the task solution, we examined performance at asymptotic levels of the initial 10-s training. A three-way ANOVA was conducted on the percentage accuracy scores over the final five sessions of training at the 10-s delay. Age (young or old) and sex (male or female) were between-subjects variables, and subtest (center–nonoption, center–correct, and center–incorrect) was a within-subject variable. There was a main effect of age, $F(1, 61) = 48.33$, $p < .01$, with the performance accuracy of young dogs being better than that of the aged dogs. There was also a main effect of subtest, $F(1.97, 119.91) = 32.66$, $p < .01$ (see Figure 4c). Post hoc comparisons revealed poorer performance accuracy on the center–incorrect subtest relative to the center–nonoption ($p < .01$) and center–correct ($p < .01$) subtests. Similar results were obtained even when analysis was restricted to only those dogs that successfully passed the initial criterion. There was a main effect of age, $F(1, 19) = 4.80$, $p < .05$, and subtest, $F(1.93, 36.70) = 24.33$, $p < .01$ (see Figure 4d). Post hoc comparisons revealed poorer performance accuracy on the center–incorrect subtest relative to the center–nonoption ($p < .01$) and center–correct ($p < .01$) subtests. Finally, we looked at the effect of prior training on the object recognition task on 3-DNMP task acquisition. A Fisher's exact test was conducted on both the young and aged group. Prior test experience did not affect 3-DNMP acquisition for either the young ($p = .21$) or the old ($p = .99$) dogs.

Position Bias

At the start of training, the majority of subjects showed a preference for one location, yielding high PBI scores. Many of the

old dogs maintained this bias throughout the period (see Figure 5). To compare the groups statistically, we conducted a two-way repeated measures ANOVA on block averages (five sessions), with PBI scores as a within-subject variable and age (young and old) as a between-subject variable. Statistically significant main effects were obtained for age, $F(1, 58) = 20.06, p < .01$, and test block, $F(9, 522) = 6.24, p < .01$. There was also a significant Age × Block interaction, $F(9, 522) = 3.25, p < .01$. As illustrated in Figure 6a, these results are due to the PBI scores of aged dogs showing slower decline during training than those of the young dogs.

When the same analysis was conducted on only the dogs that acquired the task, significant effects were obtained for age, $F(1, 17) = 6.57, p < .02$, and block, $F(9, 153) = 5.05, p < .01$, but the Age × Block interaction was not significant. Overall, these aged dogs still possessed higher PBI scores than the young dogs. However, over the course of training, PBI scores for both age groups decreased at a similar rate (see Figure 6b).

Behavioral Strategies

A qualitative analysis was conducted to examine the strategies used by young and old dogs. Some dogs displayed performance curves that averaged to chance levels of accuracy throughout training (see Figure 7a), whereas others were unable to move

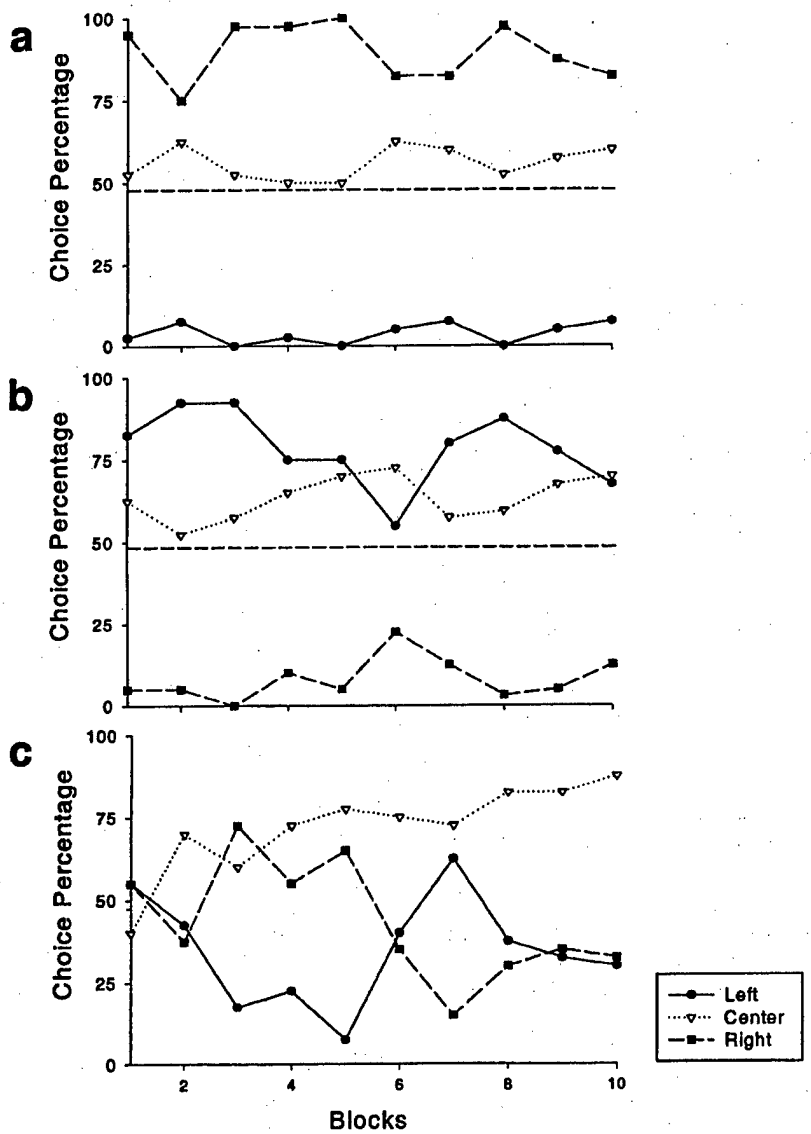

Figure 5. Position preference responding for 3 aged dogs over the course of training on the three-position delayed nonmatching-to-position task. a: A dog that started with and maintained a strong right position bias and a strong left-side avoidance. b: A dog that showed a consistent right-side avoidance. c: A dog that initially responded to all three locations but, over the course of training, developed a very strong center position bias.

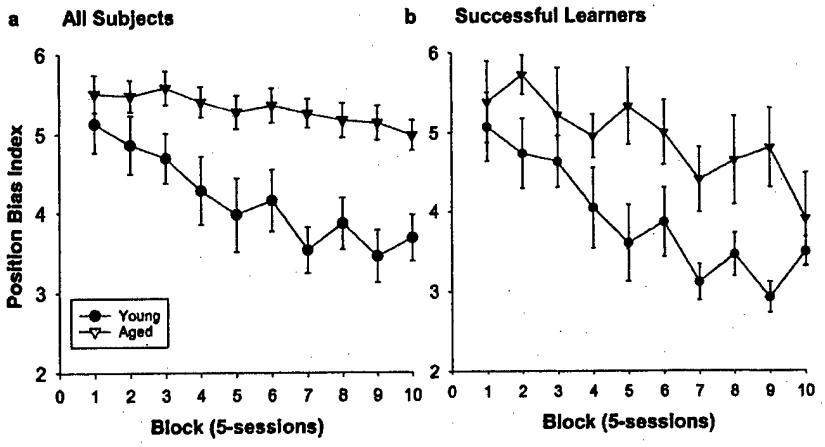

Figure 6. Age differences in position bias index (PBI) scores in the three-position delayed nonmatching-to-position task over the 50 training sessions, separated into 5-session blocks. a: PBI scores for all subjects. Only the young dogs showed progressive decline in PBI scores. b: PBI scores for only the aged and young dogs that successfully acquired the task. Both age groups showed a progressive decline in PBI scores. Error bars represent standard error.

beyond Stage 1 learning, and their performance on the center–incorrect subtest remained poor (see Figure 7b).

The dogs that acquired the task tended to do so in two stages. In the first stage, performance on the center–nonoption and center–correct subtests improved first, and in the second stage, accuracy on the center–incorrect subtest increased later (see Figures 7c and 7d). However, there were exceptions, with 4 young dogs appearing to learn the entire task in one stage; their performance accuracy on all three subtests improved coincidentally. Figure 7e shows the most dramatic illustration of this pattern. This dog performed at a chance level over the first 32 successive sessions. On the 33rd session, the subject's performance was perfect. As illustrated in Figure 7e, after initially learning, the subject maintained a high level of performance. This pattern of learning was not seen in any of the aged dogs.

To determine whether young dogs used different strategies than old dogs, the frequencies of dogs using each of the three task solutions (positional, S-R, or cognitive) was compared by means of a chi-square test. As illustrated in Figure 8a, there was a significant effect of age on the type of strategy, $\chi^2(5, N = 64) = 24.04$, $p < .01$, at the 10-s delay. A higher percentage of aged dogs used position bias strategy than young dogs, which used an S-R or cognitive strategy. Figure 8b compares strategies used by those dogs that acquired the task. In these groups, a Fisher's exact test revealed no significant age differences.

Maximal Memory Capacity

The successful dogs were ranked according to the longest delay they were able to pass within 40 sessions after acquiring the task at the 10-s delay. A Mann–Whitney U test of age by ranking revealed significant differences, $U(8, 15) = 17.50, p < .01$. Young dogs were able to perform more accurately at longer delays than the aged dogs (see Figure 9).

DNMP Task Comparison Using Historical Data

We compared acquisition on the 3-DNMP task by the young dogs to that of a similar group of young dogs on the 2-DNMP task, from our historical database previously described by Adams, Chan, Callahan, Siwak, et al. (2000). The historical sample consists of 15 young (1–3 years old) and 50 aged (8–12 years old) dogs. The dogs were all administered a standard training protocol before the study to familiarize them with the testing apparatus and procedures (Milgram et al., 1994). The standard pretraining protocol included training on reward and object approach learning, object discrimination learning, and discrimination reversal learning.

The failure rate of aged dogs was 83% on the 3-DNMP task, compared with only 18% on the 2-DNMP task. Of the young dogs, 12% failed to acquire the 3-DNMP task, whereas none failed to acquire the 2-DNMP task (Adams, Chan, Callahan, Siwak, et al., 2000).

A two-way ANOVA of sessions to pass criterion was conducted with task (2-DNMP and 3-DNMP) as a between-subjects variable and delay (10 s, 20 s, and 30 s) as a within-subjects variable. There was a significant effect of task, $F(1, 21) = 11.32$, $p < .01$, with young dogs acquiring the 2-DNMP in fewer sessions than the 3-DNMP. There was also a significant effect of delay, $F(2, 42) = 5.60$, $p < .01$, with post hoc comparisons revealing that more errors were committed during acquisition of the 10-s delay than during acquisition of the 20-s ($p < .02$) or 30-s ($p < .01$) delays. Both groups were then ranked according to their maximal memory capacity. A Mann–Whitney U test of task by ranking revealed significant difference, $U(5, 9) = 1.50, p < .01$. The dogs were able to perform more accurately at longer delays on the 2-DNMP task than on the 3-DNMP task.

Discussion

This study reexamined spatial learning and memory deficits in aged dogs with a novel DNMP task that uses three, rather than two, spatial positions. We found striking age differences in acquisition and in performance after learning: When compared with young dogs, aged dogs committed more errors, required a longer training period, and showed reduced memory capacity. The young and old

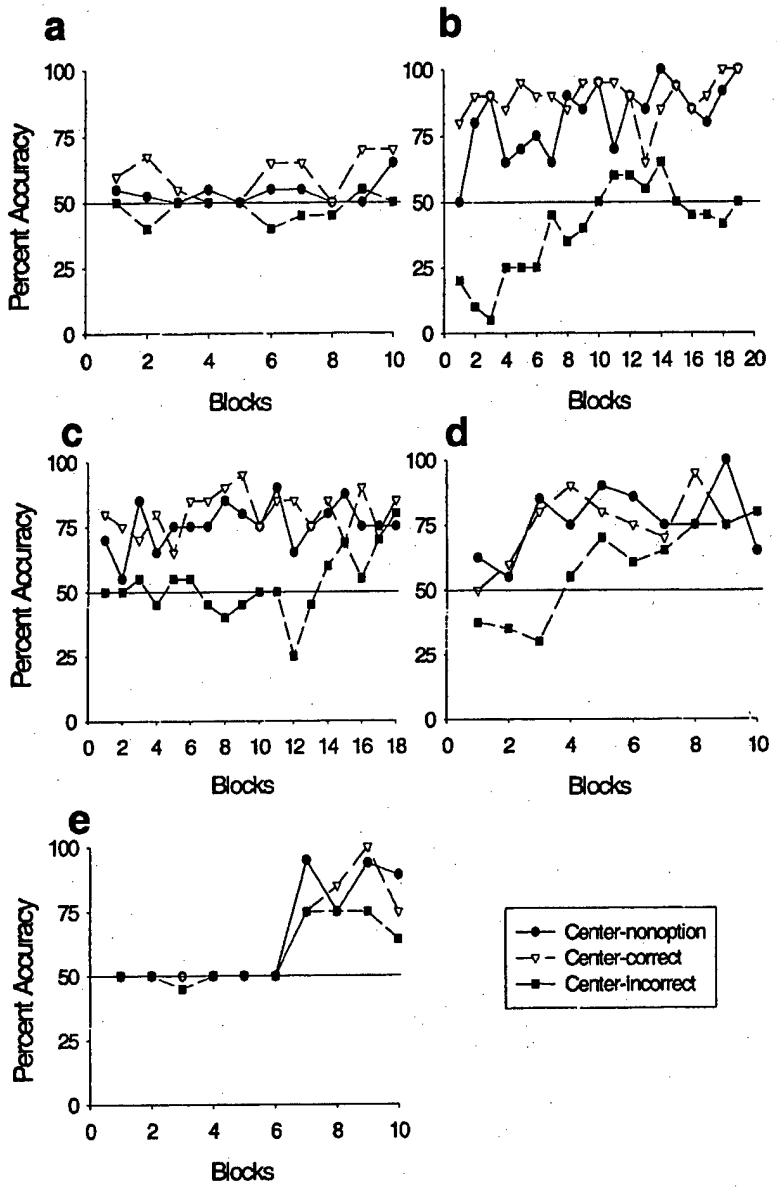

Figure 7. Shifting of strategies during learning of the three-position delayed nonmatching-to-position task over the 50 training sessions, separated into 5-session blocks. a: An aged dog that retained a position preference through the course of testing and performed at chance (50%, horizontal line) on all subtests. b: An aged dog that shifted from a positional bias to a stimulus–response (S-R) strategy, in which performance on the center–nonoption and center–correct subtests became proficient. c: An aged dog that shifted from an S-R strategy to a cognitive strategy at approximately the 14th training block, as evidenced by equal, above-chance performance on all subtests. d: A young dog that shifted to a cognitive strategy on the 4th training block. e: A young dog that acquired the cognitive strategy in a single session on all subtests.

groups came from the same colonies, but because of space limitations, the groups were housed in different facilities. This is very unlikely to have affected the outcome for three reasons. First, the testing apparatus and testing procedures were identical at both facilities. Second, these results are comparable to findings on the 2-DNMP task obtained at the University of Toronto facility, in which the failure rate was 18% for old beagles and 0% for young beagles. This contrasts with a failure rate of 12% for the young beagles on the more difficult 3-DNMP task. Third, we have now tested additional dogs, both young and old, on the 3-DNMP task at the same facility (University of Toronto) and have obtained a high success rate for young dogs and a poor success rate for old dogs.

Another noncognitive factor that could have affected the outcome of this study is differences in sensory processing ability. But

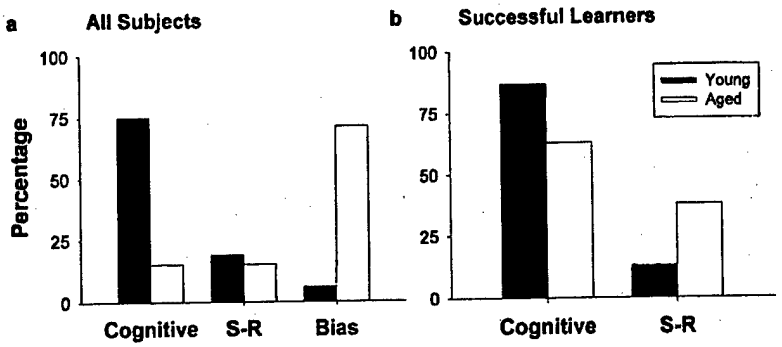

Figure 8. Age differences in the use of strategies on the three-position delayed nonmatching-to-position task. a: Percentage of dogs using a particular strategy for both age groups. The majority of aged dogs used a positional bias strategy, which is reflected in their high failure rate. b: Percentage of dogs that acquired the task by using a particular strategy. S-R = stimulus–response.

the existence of such deficits is unlikely to account for the present data. We masked the location of the food reward to prevent the dogs from using olfactory cues. The fact that the task was difficult for all of the dogs and that difficulty increased at long delays strongly suggests that the masking was effective and that dogs were not using olfactory cues. With regard to possible deficiencies in visual processing, all of the subjects had previously learned both an object discrimination and an object reversal learning task, indicating the ability to associate specific visual cues with reward.

The basic finding of age-dependent deficits in spatial learning and memory is consistent with results from the 2-DNMP task (Adams, Chan, Callahan, & Milgram, 2000; Adams, Chan, Callahan, Siwak, et al., 2000; Head et al., 1995). The magnitude of the age effect, however, was markedly larger in the present study. The majority (83%) of aged dogs failed to acquire the 3-DNMP task after 50 sessions of training. In contrast, only 18% of aged dogs failed to acquire the 2-DNMP task.

Age and Behavioral Strategies

Dogs in both age groups initially approached the task with distinct position preferences. This strategy results in reward on only 50% of the trials. Nevertheless, the majority of the aged dogs maintained a position bias strategy throughout the 50-session acquisition phase. The dogs (both young and old) that did acquire the task tended to shift first from a position strategy to an S-R

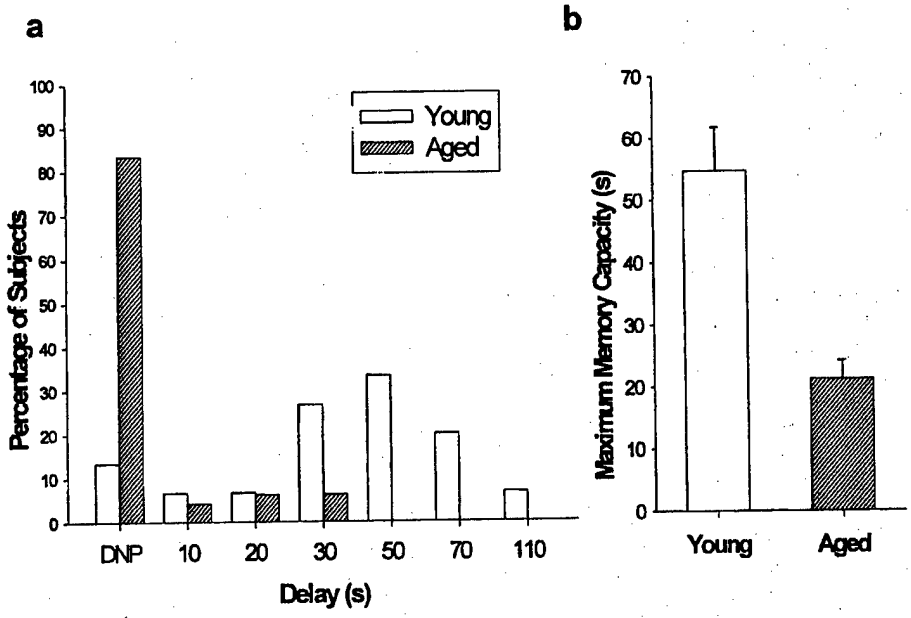

Figure 9. Age differences in maximal memory capacity (maximum delay completed) on the three-position delayed nonmatching-to-position task. a: Percentage of dogs that were able to complete each delay for both age groups. Note that the majority of aged dogs did not pass (DNP) the 10-s delay. b: Average (± *SEM*) maximal memory capacity for the dogs that learned the task.

strategy, and subsequently from an S-R to a cognitive strategy. The S-R strategy involved responding in a given direction depending on the location of the sample. This response pattern was only successful when the sample appeared at either the left or right position, that is, the center–nonoption and center–correct subtests. To perform with maximal success, the subjects had to learn the cognitive strategy, the more general rule of avoiding the sample position.

Evidence for this two-stage learning comes from an error analysis. The center–incorrect subtest was typically the last subtest to improve in performance. The aged dogs took longer to solve the center–incorrect subtest and made proportionately more errors on this subtest than did the young dogs. Both age groups showed differential performance accuracy on the subtests even over the criterion days (when they were close to asymptotic levels). Performance on the center–incorrect subtest was always poorer than on the center–nonoption and center–correct subtests. Moreover, some dogs never became proficient in solving the center–incorrect subtest and maintained the use of an S-R strategy.

We interpret the persistent use of inefficient strategies by the aged dogs as evidence of an age-dependent decline in cognitive flexibility, which can be defined as the ability to shift between problem-solving strategies. Reductions in cognitive flexibility occur with advanced age in humans (Botwinick, 1978; Daigneault, Braun, & Whitaker, 1992), rats (Stephens, Weidmann, Quartermain, & Sarter, 1985), and primates (Lai, Moss, Killiany, Rosene, & Herndon, 1995; Voytko, 1993, 1999). Cognitive flexibility is generally thought to depend on the integrity of the prefrontal cortex (Daigneault et al., 1992; Dias, Robbins, & Roberts, 1996; Gansler, Covall, McGrath, & Oscar-Berman, 1996). We have recently found that deposition of β-amyloid protein occurs in a brain region-dependent pattern in aged dogs, with the prefrontal cortex being the area affected earliest (Head et al., 2000). Although we do not currently possess direct evidence as to the neural circuitry underlying the 3-DNMP task, previous studies with dogs have shown that auditory-based delayed response performance is markedly disrupted by prefrontal cortex lesions (Lawicka & Konorski, 1959; Lawicka, Mishkin, Kreiner, & Brutkowski, 1966).

Age Differences in Maximal Memory Capacity

As previously mentioned, age also affects the ability to perform accurately at long delays. We have described similar results with the 2-DNMP task (Adams, Chan, Callahan, Siwak, et al., 2000; Head et al., 1995). Age-dependent spatial memory deficits have also been reported in nonhuman primates. However, with increasing delays, accurate performance decreases more rapidly in nonhuman primates than it does in dogs. Age differences are noted at delays as short as 5 s (Presty et al., 1987), and performance drops to chance at delays of 30 s (Bartus et al., 1978; Marriott & Abelson, 1980). Differences in testing protocols may partly account for such species differences. The nonhuman primate studies have used matching-to-sample procedures to test spatial memory, which may be more difficult for monkeys than nonmatching-to-sample procedures (Mishkin & Delacour, 1975). In contrast, the current study used a nonmatching paradigm. Furthermore, the dogs were allowed to obtain reward by responding to the sample, whereas in primate tasks, subjects are often simply shown the location of the sample.

The existence of age-dependent deficits in visuospatial memory may help to account for the deficits we obtained in learning. Aged monkeys (Bartus et al., 1978; Rapp & Amaral, 1989) and rats (Dunnett et al., 1988) are not impaired on a delayed response task when the delay is very short (< 1 s). Our relatively long 10-s training delay may have contributed significantly to the age-related acquisition deficits. The dogs must be able to remember the location of the sample before they can acquire the general nonmatch rule.

We used a 10-s training delay because we previously found that both young and old dogs readily learn the 2-DNMP task. Furthermore, aged dogs are typically able to perform accurately on the 2-DNMP task at delays of 50 s (Adams, Chan, Callahan, & Milgram, 2000; Adams, Chan, Callahan, Siwak, et al., 2000). However, the 3-DNMP task is more difficult to learn than the 2-DNMP task. In the DNMP procedure, there are only two unique configurations between sample and nonmatch that must be learned (left–right and right–left). However, the 3-DNMP comprises six unique sample–nonmatch configurations (left–right, left–center, right–left, right–center, center–right, and center–left). Trying to determine the correct relationship among these many configurations may overload the memory capacity of the aged group, that is, the amount of data that can be considered at any one time.

Individual Differences

As expected, marked individual differences were seen in both the young and old dogs. Perhaps most notable were the 4 young dogs who acquired the cognitive strategy in a single stage. This very rapid learning may reflect a higher order type of rule learning, in which the nonmatch rule was not induced out of an associatively learned behavior. The absence of this type of learning in any aged dogs may be indicative of an age-related decline in higher order cognitive abilities. Aging is associated with a decline in executive function in both humans (Grigsby, Kaye, & Robbins, 1995) and primates (Lai et al., 1995).

Conclusions and Future Utility

We have described a novel modification of a DNMP task, in which the use of an orientation strategy does not lead to maximal performance. Microanalysis of choice responses of the task proved to be extremely powerful in uncovering age-related differences in cognition by identifying various behavioral strategies. Notably, the aged dogs were markedly deficient in learning the task and showed extensive spatial memory impairments. A number of factors may contribute to the age differences found in this study, other than those directly related to visuospatial function. These include age-related decreases in behavioral flexibility and working memory. The large majority of aged dogs did not achieve the preset criterion. However, this does not mean they are incapable of learning the task. First, aged dogs may be capable of more proficient learning if the initial delay is shortened. Second, we have preliminary evidence that previous training on the 2-DNMP task can facilitate learning of the 3-DNMP in aged dogs. Finally, the number of acquisition sessions was limited to 50 in this study, and more extensive training would likely have led to a higher success rate.

Future studies should probably include an age group intermediate between the old and young groups used in the present study. It would also be of considerable interest to test both rodents and primates on a similar task. Because potential use for orienting strategies is reduced in this task, it may be particularly useful in rodent studies, in which the validity of the traditional 2-DNMP task has been questioned (Chudasama & Muir, 1997).

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Locomotor Activity Rhythms in Dogs Vary With Age and Cognitive Status

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Beagle dogs exhibited diurnal patterns of locomotor activity that varied as a function of age, cognitive status, and housing environment. Aged dogs housed in an indoor facility showed a delayed onset of activity following lights on and displayed shorter bouts of activity, with more rest periods during the day, compared with young dogs. Cognitively impaired aged dogs were more active and showed a delayed peak of activity compared with unimpaired aged dogs. Housing in continuous light did not disrupt activity rhythms. The effect of age was less prominent in dogs housed in an indoor/outdoor facility. This suggests that bright sunlight and natural light–dark transitions are better able to consolidate and synchronize the activity rhythms of the dogs.

Forty to seventy percent of the elderly population suffer from chronic problems associated with sleep (Van Someren, 2000a, 2000b). These include increased nighttime wakefulness and frequent daytime naps, both of which are indicative of disrupted circadian regulation (Hofman, 2000). Elderly people are also less sensitive to entraining cues (i.e., light), and lose synchronization with the environment (Edgar, 1994; Hofman, 2000; Van Someren, 2000a, 2000b; Weinert, 2000). These effects are exaggerated in patients with Alzheimer's disease (AD), who also experience fragmentation of the cycle, with frequent waking at night and daytime napping (Hofman, 2000; Satlin et al., 1991; Satlin, Volicer, Stopa, & Harper, 1995). Patients with AD also show a phase delay, with peak activity occurring later in the afternoon, compared with control subjects (Satlin et al., 1991, 1995). More severe disruption is associated with greater disease severity (Witting, Kwa, Eikelenboom, Mirmiran, & Swaab, 1990).

Variation also exists among AD patients; with a subgroup of individuals exhibiting pacing behavior and higher levels of activity than normal controls, whereas nonpacers are less active than normal aged individuals (Satlin et al., 1991). The alterations associated with dementia vary with the type of dementia (Harper et al., 2001). Circadian disturbances are distinct for AD, frontotemporal dementia, and multi-infarct dementia (Aharon-Peretz et al., 1991; Harper et al., 2001; Mishima et al., 1997; Satlin et al., 1991, 1995).

Age-dependent changes in activity rhythms also occur in animal models. Aged rats, hamsters, and mice show dampened activity rhythms, reduced synchronization with the environment, and greater fragmentation (Weinert, 2000). The free-running period of the circadian activity–rest rhythm shortens or lengthens with age in hamsters and rats as measured under constant lighting conditions (Asai, Ikeda, Akiyama, Oshima, & Shibata, 2000; Turek, Penev, Zhang, van Reeth, & Zee, 1995). Age-dependent disorganization of circadian rhythms in mammals has been linked to degeneration of the suprachiasmatic nucleus of the hypothalamus (SCN; McDuff & Sumi, 1985; Saper & German, 1987; Satlin et al., 1991, 1995; Swaab, Fliers, & Partiman, 1985; Tate et al., 1992; Van Someren, 2000a, 2000b; Weinert, 2000).

Circadian rhythm disturbances may contribute to age-dependent cognitive dysfunction. Impaired cognitive function has been observed in both humans and rodents subjected to intentional sleep disruption during the subjective night period. (Antoniadis, Ko, Ralph, & McDonald, 2000; Bonnet, 1989; Devan et al., 2001; Sandyk, Anninos, & Tsagas, 1991). This suggests that disruptions in circadian rhythms are linked to age-associated cognitive decline (Antoniadis et al., 2000; Sandyk et al., 1991). Disruptions in

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circadian rhythms, specifically activity–rest cycles, could affect cognition in several ways: (a) by producing proactive interference on subsequent learning (Bonnet, 1989; Downey & Bonnet, 1987); (b) by producing retroactive effects by disrupting previous learning (Devan et al., 2001; Sandyk et al., 1991); or (c) by disrupting attentional mechanisms, leading to deficits in cognitive performance (Wimmer, Hoffman, Bonato, & Moffitt, 1992).

The present experiment sought to further explore the relationship between aging, cognitive function, and locomotor activity rhythms in a novel animal model, the beagle dog. Dogs clearly exhibit activity–rest cycles (Tobler & Sigg, 1986), but to our knowledge, age-related alterations have not been examined in this species. Dogs are a viable model system to study activity–rest rhythms because of a prominent daily rhythm of melatonin levels, with higher levels of melatonin during the dark part of the cycle alternating with low levels during the light period; the existence of a retinohypothalamic projection; and the presence of the various sleep stages, slow-wave and REM sleep, like those observed in humans (Lucas, Foutz, Dement, & Mitler, 1979; Schwartz et al., 1986). Studies on activity rhythms in dogs, however, are limited to canine narcolepsy and typically use young dogs (Kaitin, Kilduff, & Dement, 1986; Lucas et al., 1979; Nishino, Tafti, Sampathkumaran, Dement, & Mignot, 1997; Schwartz et al., 1986).

We examined locomotor activity rhythms in young and old dogs housed in two different facilities by using an activity-monitoring device that was worn on a collar. Dogs were acquired from three sources, and to control for possible source effects, we only compared dogs from the same source. To directly compare the effect of the two different housing conditions, dogs from one source were split into two groups and housed at the two different facilities. Dogs showed a clear activity–rest rhythm that varied as a function of both age and test facility. Within the group of old dogs, we also found that the characteristics of the activity rhythms varied with cognitive status.

General Method

Activity patterns were monitored for 5.6 days with the Mini-Mitter Actiwatch-16 activity-monitoring system (Mini-Mitter Co., Bend, OR) adapted for dogs. The Actiwatch was placed inside a specially designed animal case and placed on a collar around the dog's neck. Subjects were allowed to follow their usual patterns of activity, rest, exercise, and feeding, and the time of each event was recorded. For the dogs housed entirely indoors, assessments were collected from Sunday to Saturday. The 5 complete days when the daily routine was similar each day were, therefore, Monday to Friday. Measures were collected between February and October as the weather does not affect the conditions in an indoor facility. Assessments at the indoor/outdoor facility were collected from Thursday through Tuesday. Thus, the 5 complete days were Friday to Tuesday. At this facility, the daily routine during the weekends is the same as during weekdays. Measures were collected between May and June to ensure that the weather conditions at this indoor/outdoor facility were the same for all of the dogs.

A measure of total activity over 24 hr was provided by the Actiware-Rhythm software included in the Mini-Mitter Actiwatch activity monitoring system. The number of minutes between lights on and activity onset were calculated manually. Activity onset was considered to be the first bout of activity lasting for a minimum of 30 min. Activity bouts beginning prior to lights on were assigned a negative value for the number of minutes. The length of the main activity bout was calculated manually as the number of hours that the longest consolidated bout of activity lasted, based on its appearance on an actogram generated by the Actiware-Rhythm software.

A forced-entry regression analysis was conducted to determine whether age accounts for significant variability in the five dependent measures, beyond that accounted for by gender or location. Step 1 entered gender, Step 2 entered location, and Step 3 entered chronological age. The dependent measures were average total activity over 24 hr, length of the main activity bout, minutes between lights on and activity onset, number of daytime activity dips, and number of nighttime activity bouts.

A second regression analysis was performed to determine whether cognitive ability accounts for significant variability in the five dependent measures, beyond that accounted for by gender. Step 1 entered gender, and Step 2 entered cognitive score. This regression was conducted only with the aged dogs from Experiment 1 because cognitive data were not available for the dogs from Experiments 2 and 3.

For Experiment 1 only, a repeated measures analysis of variance (ANOVA) was used to examine the stability of total activity over 24 hr for each of the 5 complete days of recording. Day was the repeated measure, and group was a between-subjects variable.

To examine the qualitative differences between the groups of dogs, a multivariate analysis of variance (MANOVA) was conducted for average total activity over 24 hr, time of onset of activity from lights on (minutes), length of main activity bout (hours), and frequency of daytime activity dips and nighttime bouts of activity as dependent measures for all three experiments. For Experiment 1, group (age and cognitive function) was the between-subjects variable, and Tukey's honestly significant difference test was used for multiple comparisons. Age was the between-subjects variable for Experiment 2, and location was the between-subjects variable for Experiment 3.

Activity for each hour of the 24-hr period was assessed with a two-way repeated measures ANOVA with the repeated measure of hour and the between-subjects variables of group for Experiment 1, age for Experiment 2, and location for Experiment 3. Simple main effects were performed to further examine a significant interaction.

Experiment 1: Changes in Activity Rhythms With Age and Cognitive Function in Beagle Dogs

The first experiment examined activity rhythms in young and aged dogs. We cognitively characterized the aged dogs to determine whether cognitive function is related to activity rhythms.

Method

Subjects. Forty beagle dogs were included in the study: 9 puppies, aged 4–6 months (2 males, 7 females); 15 young dogs, aged 1–4 years (9 males, 6 females); and 16 old dogs (unimpaired: 6 males, 2 females; impaired: 3 males, 5 females), aged 9–14 years. The dogs were housed in an indoor facility with a light intensity, measured on the floor of the rooms, of approximately 300 lux. The dogs were individually housed in 1.07 × 1.22-m pens and maintained on a 12-hr light–dark cycle. The puppies were group housed in one room, which was approximately the size of four individual pens. Water was available ad libitum. Dogs were fed approximately 300 grams of chow daily. The puppies received Hill's Science Diet Puppy Canine Growth Dry Food (Hill's Pet Nutrition, Topeka, KS), and all of the other dogs received Purina Dry Dog Chow (Ralston Purina, St. Louis, MO). Pens were washed daily between 9 and 11 a.m., during which time the dogs were exercised in groups in a separate room. Working hours of the laboratory personnel were from 8 a.m. to 4 p.m. All dogs were in good health at the time of behavioral testing.

Eight dogs (3 young males and 5 old females) from the indoor facility were selected for analysis of activity rhythms under constant light conditions for 5.6 days to determine whether the activity cycles would be more difficult to maintain among older dogs in these conditions.

Cognitive characterization procedures. Cognitive characterization was based on the sum of the dogs' errors on the acquisition of three

neuropsychological tests: a delayed nonmatching-to-position (DNMP) task, an object discrimination learning task, and an object discrimination reversal task (Chan et al., 2002; Milgram, Head, Weiner, & Thomas, 1994). An aged dog was considered impaired if its total score was greater than 2 *SD* from the mean of the young dog group. An aged dog with a score less than 2 *SD* from the mean of the young dogs was placed in the unimpaired group. This resulted in 8 old-unimpaired dogs (6 male, 2 female) and 8 old–impaired dogs (3 males, 5 females).

The test apparatus, as described previously (Milgram et al., 1994), consisted of a wooden box (1.150 m long × 1.080 m high × 0.609 m wide) with vertical aluminum bars at the front, a moveable Plexiglas tray with three food wells, a small overhead incandescent light, and a wooden partition containing a one-way mirror and hinged door. The heights of the vertical bars were individually adjusted for each dog to allow access to the food placed in the tray wells. A dedicated computer program was used for controlling all timing procedures, for specifying the location of the correct choice, and for capturing data. The test sessions occurred once daily.

The object discrimination task used two objects: a blue Lego block and a yellow coffee jar lid. The tray was presented, with the two objects placed over the lateral wells. The dog had to displace the object that was associated with the reward. The dogs were given 10 trials per day with a 30-s interval between trials. Dogs were tested daily until they passed. The learning measure used was errors made until criterion was reached.

The object discrimination reversal task simply changed which object was rewarded, so that the dogs had to learn to go to the previously unrewarded object. For example, if the blue Lego block was positive during the discrimination test, the yellow coffee jar lid was positive for the reversal learning task.

The DNMP task was intended to train dogs to remember the location of a sample stimulus. Each trial of the task involves two components: the sample phase and the test phase. During the sample phase, the dog was presented with an object covering a food reward in one of the three wells. The tray was then removed for a delay of 10 s. After the delay, the tray was presented a second time, with the sample object covering the same well and a new identical object covering a second well, which now contained food. Thus, the dog had to respond to the object in the new location to receive the food reward. The dogs were considered to have made an incorrect choice if they came into contact with the sample object that had previously been presented. The dogs were given 10 trials per day with a 60-s interval between trials. When the dog passed the task at the 10-s delay, it moved on to a 20-s delay and then a 30-s delay. The longer delays make the task more difficult. The learning measure used was errors made to reach criterion for each delay.

Results

The mean (± *SD*) cognitive score and standard deviation for the young dog group was 185 (± 59). Any aged dog with a score greater than 303 was classified as impaired. The distribution of error scores for the combined error score and the age of the dogs are illustrated in Figure 1.

Multiple regression analyses. The results of the overall regression analysis are listed in Table 1. Gender did not significantly account for any variability in the five dependent measures. Location significantly affected the length of the main activity bout, the time between activity onset following lights on, and the number of daytime rest periods. Chronological age was a significant predictor of total activity and accounted for additional variability in the length of the main activity bout, the time between lights on and activity onset, the number of daytime rest periods, and nighttime activity bouts.

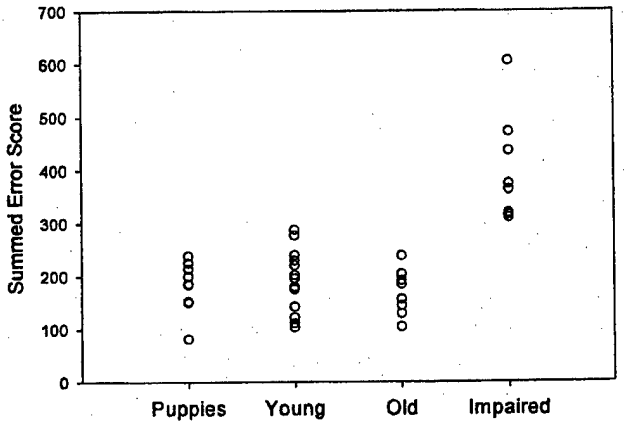


Figure 1. The combined sum of errors required to learn a delayed nonmatching-to-position task and an object discrimination and reversal task are plotted by age group in the indoor-facility dogs. Aged dogs with scores greater than 2 *SD* from the mean of the young dogs were considered impaired.

The results of the second regression analysis examining the influence of gender and cognitive score on the dependent measures are listed in Table 2. Again, gender did not significantly account for any variability in the dependent measures. Cognitive score accounted for variability in total activity averaged over 5 days, the length of the main activity bout, and the number of daytime rest points.

On the basis of the regression analyses results, the data were analyzed separately for each location, and gender was not included as a variable. Our goal was to assess the qualitative nature of the effects of age, cognitive ability, and location.

Interday stability of total activity. The repeated measures ANOVA indicated that the total activity over the 24 hr of each day did not differ between the 5 complete days of recording, $F(4, 144) = 0.43$, $p = .78$. Daily activity was stable over the 5 days. The effect of group was significant, $F(3, 36) = 81.39$, $p < .01$. The puppies were significantly more active than the old, old–impaired, and young dogs ($p < .01$). Old–unimpaired dogs were less active than the old–impaired ($p = .02$) and young dogs ($p < .01$).

Effect of age and cognition on activity level. Dogs exhibited clear activity rhythms, with higher levels of activity during the day in all four groups of dogs (Figure 2). For the total activity measure averaged across the 5 days of recording, the MANOVA revealed a significant effect of age group, $F(3, 36) = 123.66$, $p < .01$. Overall, the puppies ($M = 454.44$, $SEM = 17.33$) were more active than the young dogs ($M = 145.75$, $SEM = 12.47$; $p < .01$), old–unimpaired dogs ($M = 80.07$, $SEM = 5.10$; $p < .01$), and old–impaired dogs ($M = 141.99$, $SEM = 18.17$; $p < .01$). The old–unimpaired dogs were also less active compared with the young ($p = .01$) and old–impaired dogs ($p = .05$). Differences between the groups of dogs were apparent during the daytime hours when activity levels were higher than at night; the groups did not differ during the nighttime.

The ANOVA for the hour-by-hour analysis revealed significant main effects of age group, $F(3, 36) = 81.39$, $p < .01$, and hour, $F(23, 828) = 96.03$, $p < .01$. The interaction between group and

Table 1
Summary of Multiple Regression Analysis for Gender, Location, and Chronological Age

Variable	F	p	R^2	R^2 change
Total activity over 24 hr				
Model 1: Gender	1.967	.165	.025	.025
Model 2: Gender, location	1.867	.162	.047	.022
Model 3: Gender, location, age	14.887	**.000**	.373	.326
Length of main activity bout				
Model 1: Gender	0.047	.830	.001	.001
Model 2: Gender, location	4.228	**.018**	.100	.100
Model 3: Gender, location, age	21.132	**.000**	.458	.358
Activity onset from lights on				
Model 1: Gender	0.198	.657	.003	.003
Model 2: Gender, location	6.832	**.002**	.152	.150
Model 3: Gender, location, age	19.091	**.000**	.433	.281
Daytime activity dips				
Model 1: Gender	0.597	.442	.008	.008
Model 2: Gender, location	7.284	**.001**	.161	.153
Model 3: Gender, location, age	8.182	**.000**	.247	.086
Nighttime activity bouts				
Model 1: Gender	2.000	.161	.025	.025
Model 2: Gender, location	3.006	.055	.073	.048
Model 3: Gender, location, age	3.312	**.024**	.117	.044

Note. Values in boldface represent statistically significant effects.

hour was also significant, $F(69, 828) = 11.59$, $p < .01$. For the simple main effects, one-way ANOVAs were performed to compare the groups of dogs at each hour to determine when activity levels were significantly different (Figure 3). Hour (H)1 was from 12 a.m. to 1 a.m.

The puppies were significantly more active than the young dogs at H1–2 ($p < .05$), H8–21 ($p < .05$), and H24 ($p < .01$). They were more active than the old dogs at H1 ($p < .01$), H8–22 ($p < .01$), and H24 ($p < .01$). The puppies were also more active than the old–impaired dogs at H1 ($p < .01$), H8–21 ($p < .01$), and H24 ($p < .01$). The puppies did not differ from the other groups from H3 until H7 (approximately 2 a.m. to 7 a.m.).

The young dogs were significantly more active than the old dogs at H10–12 ($p < .05$), which corresponds to 9 a.m. until 12 p.m. Young dogs were more active than the old–impaired dogs at H11 ($p < .01$). The old–impaired dogs exhibited significantly higher levels of activity than the old dogs at H15–17 ($p < .05$), corresponding to 2 p.m. until 5 p.m.

Effect of age and cognition on activity onset, length of main activity bout, daytime rest, and nighttime activity. The time of onset of activity from lights on revealed a main effect of group, $F(3, 36) = 10.22$, $p < .01$. The puppies ($M = 18.18$, $SEM = 7.32$) and young dogs ($M = 35.51$, $SEM = 9.79$) became active sooner after the room lights came on than the old–unimpaired ($M = 120.23$, $SEM = 20.97$; $ps < .01$) and old–impaired ($M = 127.98$, $SEM = 28.53$; $ps < .01$) dogs. Several of the puppies and young dogs exhibited anticipatory activity just prior to when the lights came on in their room.

Table 2
Summary of Multiple Regression Analysis for Gender and Cognitive Score

Variable	F	p	R^2	R^2 change
Total activity over 24 hr				
Model 1: Gender	3.856	.070	.216	.216
Model 2: Gender, cognitive score	4.927	**.026**	.431	.215
Length of main activity bout				
Model 1: Gender	2.704	.122	.162	.162
Model 2: Gender, cognitive score	4.660	**.030**	.418	.256
Activity onset from lights on				
Model 1: Gender	0.661	.430	.045	.045
Model 2: Gender, cognitive score	0.461	.641	.066	.021
Daytime activity dips				
Model 1: Gender	0.958	.344	.064	.064
Model 2: Gender, cognitive score	4.885	**.026**	.429	.365
Nighttime activity bouts				
Model 1: Gender	0.901	.359	.060	.060
Model 2: Gender, cognitive score	0.727	.502	.101	.040

Note. Values in boldface represent statistically significant effects.

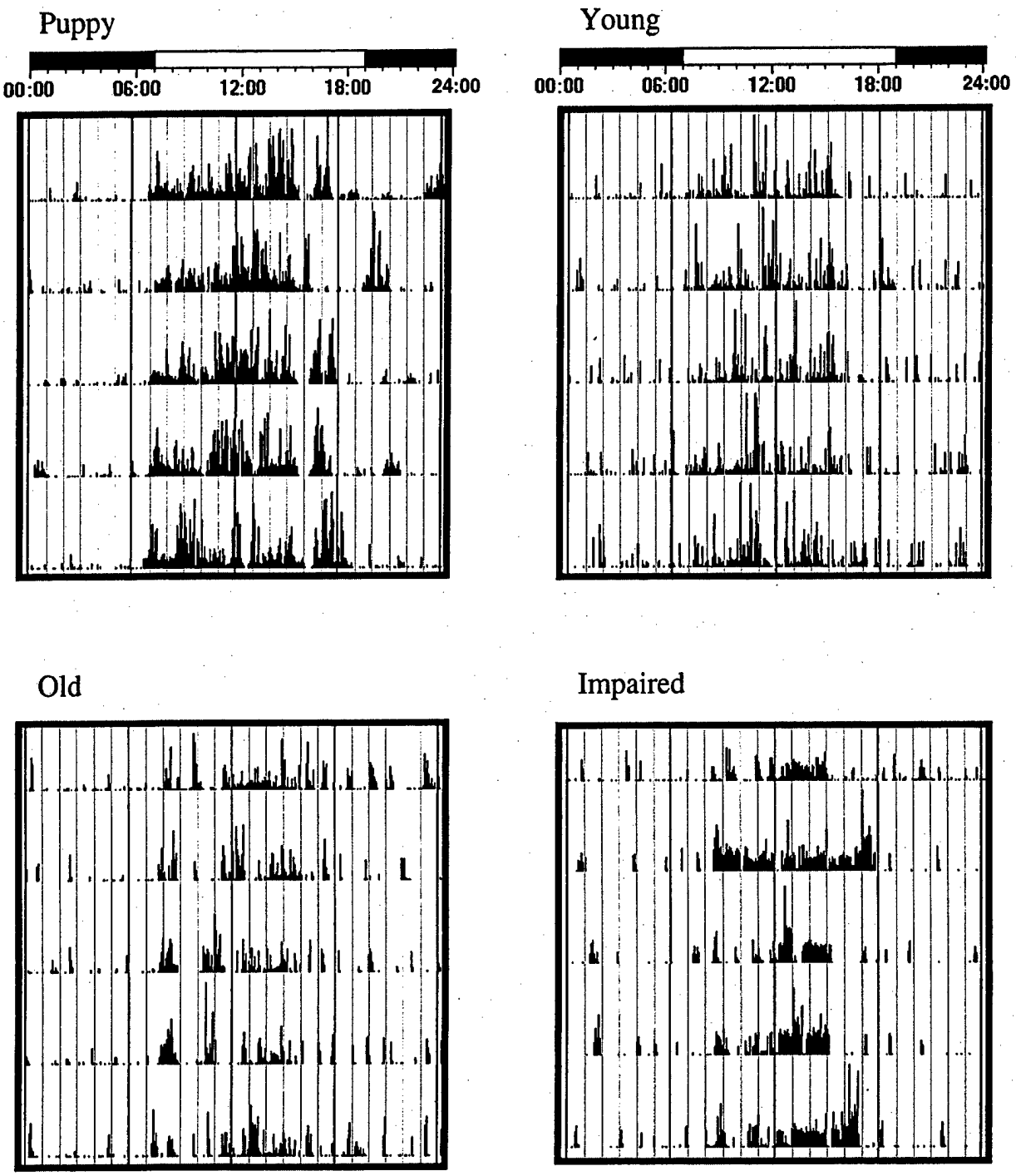


Figure 2. Computer-generated actograms for a representative dog from each group in the indoor-facility dogs. Each actogram plots the activity counts of the dog for each day of recording and the light–dark cycle (Days 1–5, top to bottom in each plot). All dogs exhibit clear activity–rest cycles, with activity being highest during the daytime hours. Differences appear in the time between lights on and onset of activity, the length of the main activity bout, and the number of daytime rest periods.

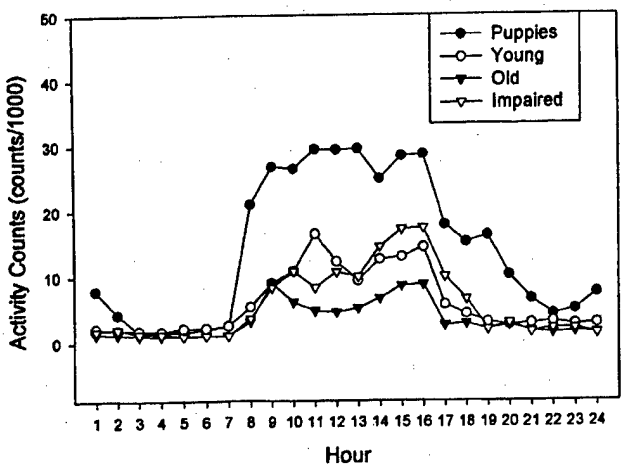


Figure 3. Mean activity count across the 5 days of recording is plotted for each hour of a 24-hr period for the indoor facility dogs: puppies, young, old, and old–impaired. Differences between the groups appear in the pattern of activity. Young dogs are most active in the morning, whereas age-impaired dogs are mainly active in the afternoon. The puppies exhibit high levels of activity during most of the day, whereas the old dogs display low levels of activity throughout the light period.

The length of the main bout of activity was significantly different between the groups, $F(3, 36) = 15.55$, $p < .01$. The puppies ($M = 8.57$, $SEM = 0.47$) and young dogs ($M = 8.71$, $SEM = 0.48$) were active for longer periods of time during the day than both old ($M = 4.51$, $SEM = 0.30$; $ps < .01$) and old–impaired ($M = 5.51$, $SEM = 0.74$; $ps < .01$) dogs.

A significant main effect of group on the number of rest points during the day was obtained, $F(1, 36) = 4.89$, $p < .01$. The old dogs ($M = 2.93$, $SEM = 0.42$) had significantly more rest points during the day than the young dogs ($M = 1.36$, $SEM = 0.25$; $p < .01$) and puppies ($M = 1.42$, $SEM = 0.16$; $p = .02$). The impaired dogs ($M = 1.80$, $SEM = 0.44$) did not differ from any group.

The number of nighttime activity bouts also produced a significant main effect of group, $F(1, 36) = 4.59$, $p < .01$. The puppies ($M = 1.62$, $SEM = 0.12$) had significantly fewer bouts of activity during the night than the young ($M = 4.51$, $SEM = 0.49$; $p < .01$) and old ($M = 4.43$, $SEM = 0.98$; $p = .03$) dogs. Impaired aged dogs ($M = 3.80$, $SEM = 0.77$) did not differ from the other groups.

Light–dark cycle versus constant light conditions. Changing the light–dark cycle to constant light conditions for a period of 5.6 days did not produce notable behavioral changes. Two dogs, 1 young male and 1 old female, exhibited higher levels of activity in constant light conditions. The remaining dogs showed no difference in activity levels in the constant light than in the light–dark condition.

All of the dogs exhibited regular patterns of daily activity during the constant light condition. Initially the patterns were synchronized with the previous light–dark cycle but exhibited a phase delay toward the end of the week (see Figure 4). There were no apparent age differences in the response to constant light conditions. There were no significant differences in the period of the cycle between the light–dark (young, 23.65 ± 0.19; old, 24.01 ± 0.14) and constant light (young, 23.85 ± 0.25; old, 24.13 ± 0.05) conditions, $F(1, 6) = 1.33$, $p = .29$.

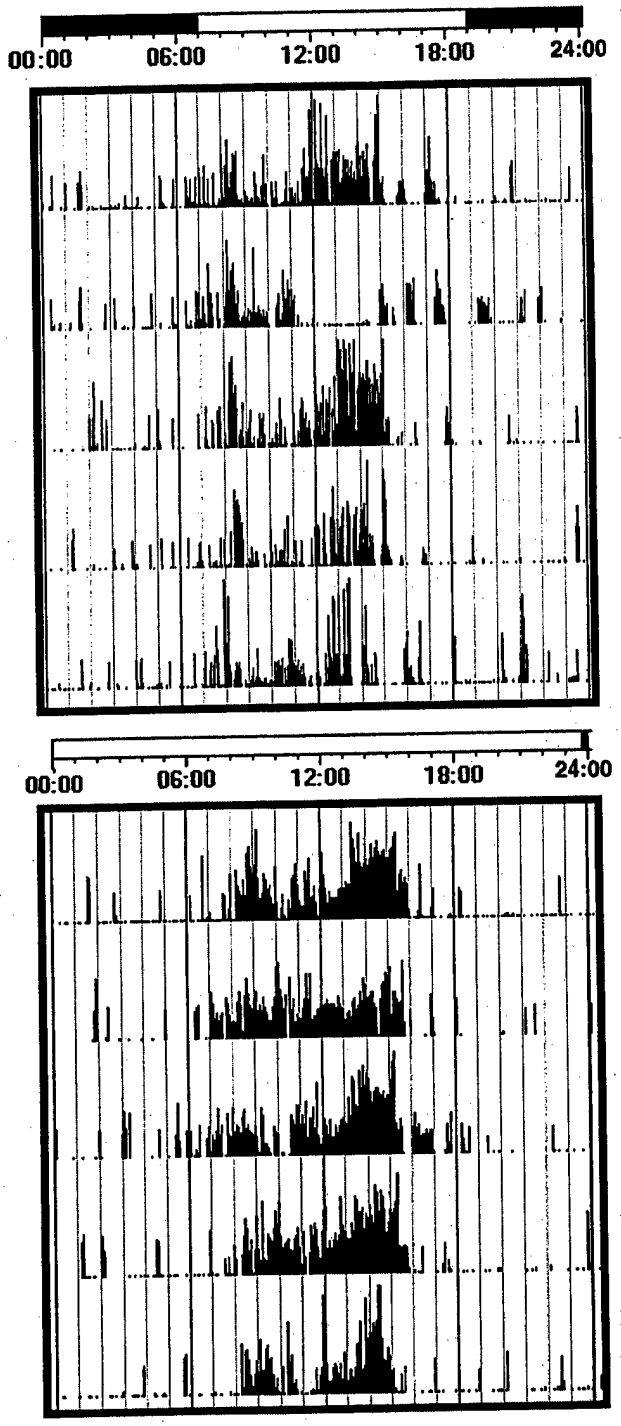


Figure 4. Computer-generated actograms of activity recordings of an indoor-facility dog in the standard light–dark cycle (top) and later in a constant-light situation (bottom). Each actogram plots the activity counts of the dog for each day of recording (Days 1–5, top to bottom in each plot). No differences were evident in the activity rhythm except for the development of a phase delay toward the end of recording in the constant-light situation.

Experiment 2: Activity Rhythms and Age in Beagle Dogs in an Indoor/Outdoor Housing Facility

This study examined differences in activity rhythms in young and aged beagle dogs housed in a facility in which part of the housing cage was indoors and a larger portion was outdoors.

Method

The beagle dogs were born at the facility and consisted of 11 old dogs aged 12–14 years (5 males, 6 females) and 8 young dogs aged 3–4 years (4 male, 4 female). These dogs were housed in natural light in an indoor/outdoor kennels (sunlight intensity is approximately 10,000 lux). The light–dark cycle was determined by the rising and setting of the sun and was approximately 14 hr light:10 hr dark. The dogs were housed either individually or in pairs in kennel buildings with indoor/outdoor runs measuring 6.10 m long × 0.94 m wide. The dog kennels were cleaned between 8 and 10 a.m., and the dogs did not leave the kennel during cleaning. The aged dogs were fed approximately 300 g of Hill's Science Diet Canine Senior Dry Food once a day, with water available ad libitum from a wall spout. The young dogs received Hill's Science Diet Canine Maintenance Dry Food. Working hours of the laboratory personnel were from 8 a.m. to 4 p.m. All dogs underwent clinical examinations for general health prior to the start of any study, and all dogs included in the study were in good health.

Results

Effect of age on levels of activity. The MANOVA revealed that the average total activity was significantly higher in young dogs (M = 308.43, SEM = 25.17) than old dogs (M = 159.13, SEM = 11.71), $F(1, 17) = 10.48$, $p < .01$.

For the hour-by-hour analysis, the ANOVA revealed significant main effects of age, $F(1, 17) = 10.06$, $p < .01$; hour, $F(23, 391) = 42.08$, $p < .01$; and a significant Age × Hour interaction, $F(23, 391) = 4.67$, $p < .01$. The one-way ANOVAs for the simple main effects analysis indicated that young dogs were significantly more active than the old dogs at H7–11 ($p < .01$), H15 ($p = .04$), and H21 ($p = .01$; see Figure 5). Actograms are shown in Figure 6.

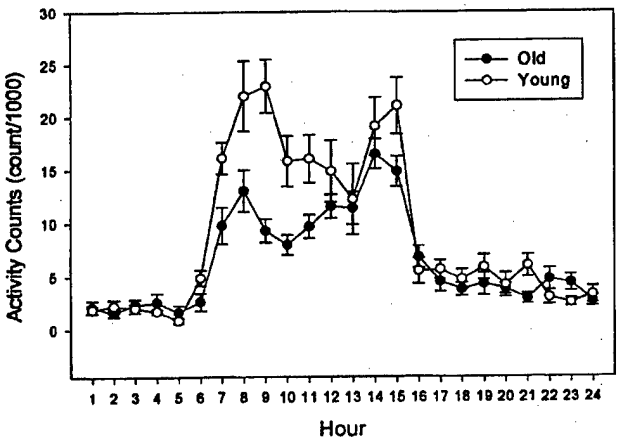


Figure 5. Mean (± *SEM*) activity count across the 5 days of recording is plotted for each hour of a 24-hr period for the indoor/outdoor-facility young and aged dogs. Young dogs were more active than old dogs during most of the daytime hours.

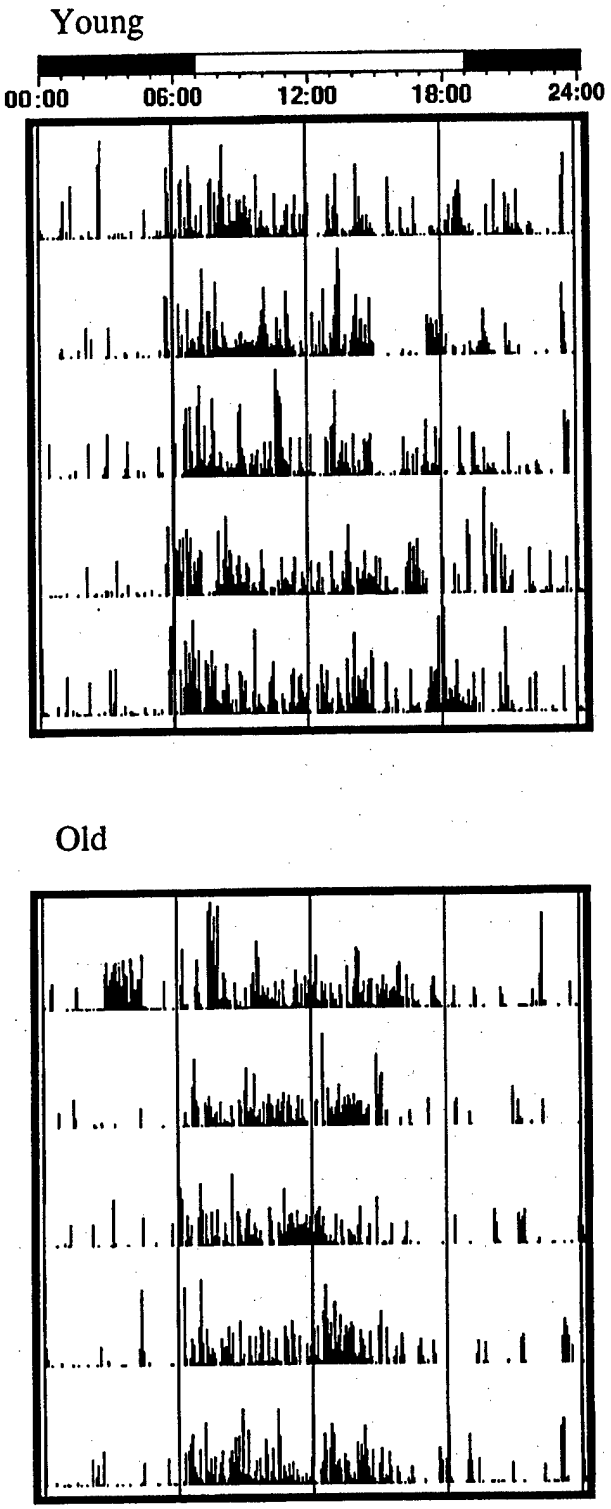


Figure 6. Computer-generated actograms for a representative dog from the young and old age groups in the indoor/outdoor-facility dogs. Each actogram plots the activity counts of the dog for each day of recording and the light–dark cycle (Days 1–5, top to bottom in each plot). All dogs exhibit clear activity–rest cycles, with activity being highest during the daytime hours.

Effect of age on activity onset, length of activity bouts, daytime rest, and nighttime activity. Young (M = 11.08 min, SEM = 5.74) and old (M = 31.75 min, SEM = 9.51) dogs did not significantly differ in the amount of time between lights on (sunrise) and activity onset, $F(1, 17) = 3.22$, $p = .09$, although the average number of minutes was higher in the old-dog group. The length of the main bout of activity did not differ, $F(1, 17) = 0.24$, $p = .63$, between the young (M = 8.86 hr, SEM = 0.61) and old (M = 8.50 hr, SEM = 0.44) dogs.

There were no significant differences, $F(1, 17) = 0.89$, $p = .36$, between the young and old dogs for the number of rest points during the day (young, M = 1.10, SEM = 0.17; old, M = 1.47, SEM = 0.31) or activity bouts during the night, $F(1, 17) = 0.37$, $p = .55$ (young, M = 2.55, SEM = 0.66; old, M = 2.95, SEM = 0.28).

Experiment 3: Comparison of Activity Rhythms in an Indoor Facility and an Indoor/Outdoor Facility in Aged Beagle Dogs

For a more direct comparison of the indoor and indoor/outdoor facilities, we examined the activity cycles of aged dogs originally from a colony where indoor rearing was practiced. One group of dogs was sent to the indoor/outdoor facility and a second group to the entirely indoor facility. This allowed us to compare the effect of facility location and design on activity rhythms in dogs that came from an indoor rearing facility.

Method

Twenty beagle dogs were obtained from a colony that practiced indoor rearing, to directly compare the effects of indoor and outdoor housing so that source effects would be eliminated. Twelve old dogs aged 10–13 years (6 male, 6 female) were transferred to the indoor/outdoor colony, and 8 old dogs aged 9–13 years (2 males, 6 females) were transferred to the entirely indoor facility. At the original facility, the dogs were housed indoors with a 12-hr light–dark. Lights were on from 6 a.m. to 6 p.m. The dogs were transferred to the new facilities at least 1 year prior to the collection of activity-monitoring data.

Results

Effect of location on levels of activity. The results of the MANOVA indicated that the aged dogs housed at the indoor/outdoor facility (M = 240.36, SEM = 29.14) were significantly more active than the aged dogs housed at the indoor facility (M = 136.47, SEM = 11.82), $F(1, 18) = 7.78$, $p = .01$.

The hour-by-hour analysis obtained significant main effects of location, $F(1, 18) = 8.74$, $p < .01$, and hour, $F(23, 414) = 30.97$, $p < .01$. The interaction between location and hour was also significant, $F(23, 414) = 9.91$, $p < .01$. The simple main effects one-way ANOVAs showed that the dogs housed at the indoor/outdoor facility were significantly more active than their counterparts at the indoor facility at H6–8 ($p < .01$), H11–14 ($p < .01$), and H16 ($p < .01$; see Figure 7). Actograms are illustrated in Figure 8.

Effect of location on activity onset, length of activity bouts, daytime rest, and nighttime activity. The amount of time between lights on and activity onset was significantly shorter, $F(1, 18) = 10.41$, $p < .01$, in the dogs housed at the indoor/outdoor facility (M = 19.00, SEM = 4.64) than those at the indoor facility (M = 121.90, SEM = 38.96). The length of the main activity bout was significantly longer, $F(1, 18) = 24.45$, $p < .01$, for the dogs housed at the indoor/outdoor facility (M = 7.46, SEM = 0.47) than for the dogs at the indoor facility (M = 3.99, SEM = 0.49).

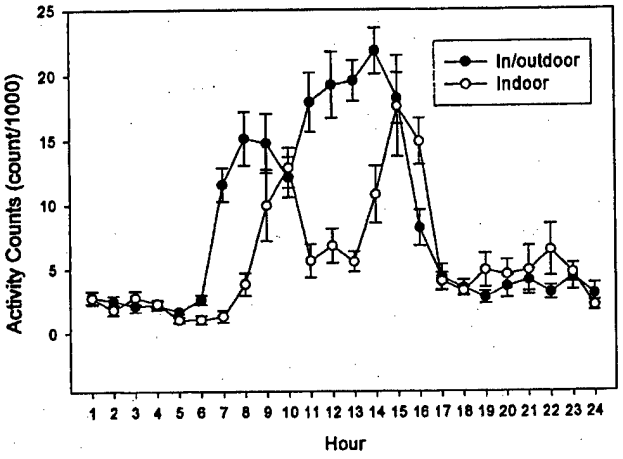


Figure 7. Mean (± SEM) activity count across the 5 days of recording is plotted for each hour of a 24-hr period for the dogs at the indoor/outdoor facility and at the indoor facility. The dogs housed partially outdoors exhibited higher levels of activity during most of the daytime hours.

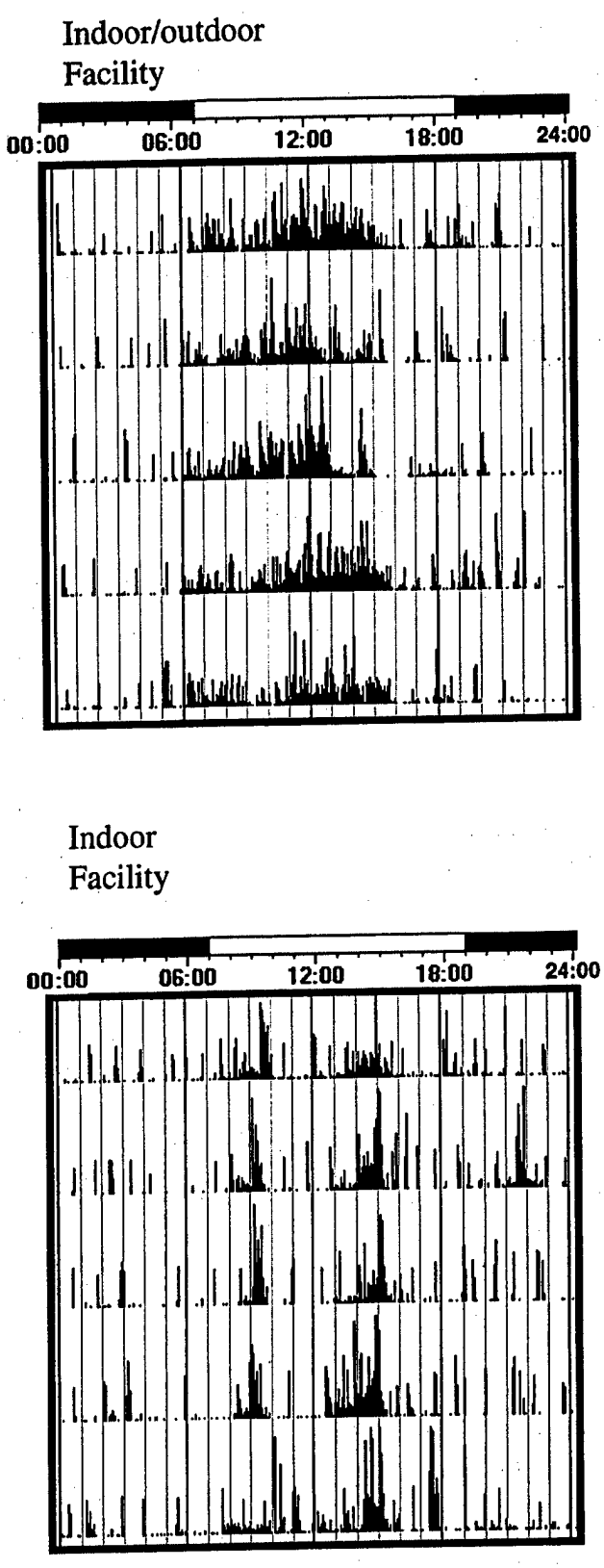
Location had a significant effect on the number of daytime rest periods, $F(1, 18) = 23.12$, $p < .01$, but not on nighttime activity bouts, $F(1, 18) = 2.90$, $p = .11$. The dogs at the indoor facility (M = 2.35, SEM = 0.46) exhibited more rest periods during the day than the dogs at the indoor/outdoor facility (M = 0.43, SEM = 0.12). The dogs at the indoor/outdoor facility (M = 3.20, SEM = 0.28) and at the indoor facility (M = 4.00, SEM = 0.39) did not differ for nighttime activity bouts.

General Discussion

This study demonstrates that beagle dogs exhibit clear activity–rest rhythms, which vary as a function of age, cognitive status, and housing environment. Activity levels were high during the day and low at night. The activity rhythms of the dogs did not merely reflect the presence of exogenous triggers; in some instances dogs became active before the lights came on and before the laboratory staff started work.

Activity Rhythms and Aging

Age-related differences are present in activity rhythms, but their manifestation depends on the housing environment. Experiment 1 demonstrated age differences in the activity–rest cycles in dogs housed in an indoor facility. At the indoor facility, the younger dogs became active earlier in the day and displayed longer, more consolidated bouts of activity than the aged dogs. The results were not replicated in Experiment 2, in which the dogs were housed in an indoor/outdoor environment. In a natural-light situation (i.e., outdoors), the onset of activity and length of time active was not different between young and aged dogs. The differences are probably linked to levels of brightness, with the outdoor environment



providing more intense light. The bright light of the sun seems to be more effective in synchronizing and consolidating the activity cycle than standard indoor lightbulbs. The results of Experiment 3 support this supposition. The dogs transferred to the outdoor facility were active earlier and exhibited more consolidated bouts of activity than their indoor counterparts. The differences between the indoor and outdoor housing situation were consistent with the other two colonies of dogs.

These results therefore suggest that age differences in activity rhythms vary as a function of light intensity. Dogs housed outdoors in natural light conditions showed fewer age differences in features of the activity rhythms than dogs housed indoors. An effect of light intensity is also reported in AD patients. Satlin, Volicer, Ross, Herz, and Campbell (1992) found that higher intensity light is able to ameliorate the alterations in the activity–rest pattern that occur in AD patients. Furthermore, Yamadera et al. (2000) found that bright-light therapy improved the cognitive state of mildly demented AD patients, and Kelly et al. (1997) found that bright light had beneficial effects in normal young men. The most likely explanation for these effects is that higher intensity light is better able to synchronize the activity–rest cycle through the retinohypothalamic tract, which connects the eye to the SCN. Light transmission through the eye and sensitivity of the visual system to light decline with age (Van Someren et al., 1996). In AD patients, degeneration of the optic nerve and retinal ganglion cells has been reported. Thus, entrainment is likely to be hampered by a reduction in perceived environmental light (Van Someren et al., 1996).

Another factor is the type of transition from light to dark between day and night. The natural light–dark cycle of the outdoors has gradual transitions between light and darkness at dawn and dusk. The indoor environment has abrupt transitions between light and dark. Kavanau (Moore-Ede, Sulzman, & Fuller, 1982) found that adding the gradual light–dark transitions to laboratory light–dark cycles increased the strength of entrainment. It is not clear why, but this supports the argument that gradual transitions contributed to the stronger entrainment to the light–dark cycle of the dogs that were housed in the outdoor facility.

At the indoor facility, the aged dogs exhibited significantly more periods of daytime inactivity than the puppies, young dogs, and impaired dogs. This probably reflects an increase in the number of naps during the day. No differences were observed at night, except that the puppies experienced fewer bouts of activity than the other groups. If one dog wakes up and barks during the night, the other dogs will be disturbed because of the housing arrangement, in which all the dogs were in the same area and could hear the other dogs. During the day, a dog taking a nap is not going to affect the other dogs. Thus, the activity cycle seems to be fragmented in the aged dogs, but not the impaired dogs. Reduced fragmentation of the cycle in the impaired dogs could be due to the higher levels of

Figure 8. Computer-generated actograms for a representative dog from each location. Each actogram plots the activity counts of the dog for each day of recording and the light–dark cycle (Days 1–5, top to bottom in each plot). All dogs exhibit clear activity–rest cycles, with activity being highest during the daytime hours. Differences between the locations appear in the time between lights on and onset of activity, the length of the main activity bout, and the number of daytime rest periods.

activity of this group, as activity itself can induce organization of the activity cycle (Hastings, Duffield, Smith, Maywood, & Efling, 1998).

Activity Rhythms and Cognition

Experiment 1 revealed that cognitive status also correlated with the activity–rest rhythm. Cognitively impaired aged dogs were more active than the normal aged dogs, primarily during the afternoon. The impaired aged dogs showed a delay in peak activity compared with the other dogs, similar to the phase delay observed in AD patients (Satlin et al., 1991, 1995). The impaired dogs were also significantly more active than unimpaired dogs as measured by the Actiwatch system in the home cage. We previously reported that cognitively impaired dogs were more active than unimpaired aged dogs in the open-field test (Siwak, Tapp, & Milgram, 2001). Satlin et al. (1991) also found that a subgroup of AD patients, called *pacers*, exhibit higher levels of activity than normal aged individuals and AD nonpacers. The present results parallel those observed in dementia, in which the timing mechanism of demented individuals appears to be compromised.

Cycle disruption in AD patients could be linked to senile plaques and neurofibrillary tangles, which develop in the SCN, the endogenous clock (Swaab et al., 1985; Van Someren, 2000a, 2000b). Age-related neuropathology in the SCN of dogs has not been examined to our knowledge, except for reports of increased reactivity to stress (Reul, Rothuizen, & de Kloet, 1991; Rothuizen, Reul, Rijnberk, Mol, & de Kloet, 1991; Rothuizen et al., 1993). Dogs show neuropathology similar to that of AD patients in other areas of the brain (Head, McCleary, Hahn, Milgram, & Cotman, 2000), suggesting that the SCN in impaired dogs may undergo changes similar to those observed in AD.

Activity Levels

An age-associated decline in motor activity has been reported in several species, including humans (Bassey, 1998; Hillerås, Jorm, Herlitz, & Winbald, 1999; LeWitt, 1988), mice (Dean et al., 1981; Elias, Elias, & Eleftheriou, 1975; Elias & Redgate, 1975; Goodrick, 1975; Ingram, London, Waller, & Reynolds, 1983; Lamberty & Gower, 1990, 1991; Rosenthal & Morley, 1989; Sprott & Eleftheriou, 1974), rats (Dorce & Palmero-Neto, 1994; Goodrick, 1971; Kametani, Osada, & Inoue, 1984), and monkeys (Emborg et al., 1998; Gerhardt et al., 1995). Siwak, Murphey, Muggenburg, and Milgram (2002) demonstrated an age-related decrement in locomotor activity in dogs using an observational test of the home cage. The present data, based on the Actiwatch, support this finding, indicating that young dogs are more active than old dogs. The differences in activity were only observed during the daytime hours, not at night.

The size of the housing area and type of facility (indoor vs. partially outdoor) influenced the level of activity of the dogs. The housing cages were 1.31 m^2 at the indoor facility and 5.73 m^2 at the indoor/outdoor. The smaller housing area limits the movement of the dogs compared with the larger kennel runs. Dogs housed in the larger runs, which were partially outdoors, displayed higher levels of activity compared with the dogs in the smaller, indoor areas. Tobler and Sigg (1986) reported that motor activity of dogs is influenced by the type of housing enclosure, with larger housing areas leading to reduced activity. The larger housing enclosures in that study, however, were indoors and isolated from laboratory activities. In the present study, the larger housing areas were partially outdoors, exposing the dogs to a variety of stimuli not present in an indoor facility. The dogs housed outdoors were also exposed to a longer light period, perhaps allowing them to be active for a longer period of time. This indicates that both the size of the housing area and exposure to the outdoors affect the motor activity of dogs.

The puppies at the indoor facility were housed in larger enclosures and displayed higher levels of activity than all of the other groups of dogs. During the morning hours of 9–11 a.m., however, all of the dogs were placed into these conditions during cleaning. The dogs were placed in a large room in groups while their housing areas were washed. This equates the conditions of all of the groups, and the younger dogs, both puppies and the young dog group, were more active than the aged groups. Thus, although the activity levels of the puppies may have been elevated because of their differential housing condition, the age difference was still apparent when all dogs were placed in the same conditions.

Activity Rhythms in Dogs

Dogs are capable of adapting their activity rhythms to their living environment. Feral dogs show higher levels of activity at night than during the day (Scott & Causey, 1973). Pet owners frequently report sleep disturbances in their dogs, but these data are based on the perception of the owner and have not been assessed experimentally in controlled environmental settings. The activity–rest cycle of dogs is probably adaptable to their living conditions, and differences are likely among feral dogs, laboratory dogs, and pet dogs. Feral dogs are active when it is safe, laboratory dogs are influenced by the constant routine and daily activity of the facility, and the activity of pet dogs will largely depend on their owners' schedules.

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Learning ability in aged beagle dogs is preserved by behavioral enrichment and dietary fortification: A two year longitudinal study

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Abbreviated Title

Antioxidants and Behavioral Enrichment Slows Cognitive Decline in Dogs

ABSTRACT

The effectiveness of two interventions, dietary fortification with antioxidants and a program of behavioral enrichment, was assessed in a longitudinal study of cognitive aging in beagle dogs. A baseline protocol of cognitive testing was used to select 4 cognitively equivalent treatment groups (Group C-C (control food - control environment), group C-E (control food - enriched environment); Group A-C (antioxidant fortified food - control environment); Group A-E (antioxidant fortified food - enriched environment). We also tested two groups of young dogs, one of which received the control food and the other, the fortified food. All of the young dogs received a program of behavioral enrichment. Discrimination learning and reversal was assessed at baseline using an object discrimination learning task, after one year of treatment condition with a size discrimination task, and again after two years with a black/white discrimination task. The performance of the four aged groups was comparable at baseline. At one year, as described previously, and at two years, the combined treatment group showed more accurate learning than any of the other groups on the discrimination and reversal learning tasks. Discrimination learning was significantly improved by behavioral enrichment. Reversal learning was improved by both behavioral enrichment and dietary fortification. By contrast, the fortified food had no effect on the performance of the young dogs as baseline results were similar to those obtained after two years of treatment. These results suggest that behavioral enrichment or dietary fortification with antioxidants and mitochondrial cofactors over a long-duration can slow age-dependent cognitive decline, and that the two treatments together are more effective than either alone in older dogs.

1. Introduction

Over the past several years, our laboratories have been studying a novel model of cognitive aging, that of the aged beagle dog. We have previously established that dogs show marked age-dependent decline in learning and memory, which varies as a function of task (Adams et al.,2000 ; Chan et al., 2002; Milgram et al., 1994; Milgram et al., 2002; Tapp et al., 2003a; Tapp et al., 2003b). The pattern of cognitive decline mirrors that seen in humans in several respects (Adams et al.,2000b). Aged dogs also develop neuropathology that is similar to that seen in both successfully aging humans and in patients with Alzheimer's Disease. Like humans, beta amyloid protein is deposited in the aging dog brain (Cummings et al. 1993; Giacconi et al., 1990), and shows a selective brain distribution that changes as a function of age (Head et al., 2000).

To date, our analysis of canine cognitive aging has been restricted to cross sectional studies, in which selected groups of aged animals are compared with selected groups of younger animals. This strategy has limitations, which include cohort effects and the possibility of selective bias in mortality. Cohort effects may occur because of factors extrinsic from aging, which could result in scores that are either particularly low, or particularly high (see Rowe and Kahn, 1987). In primate aging studies, for example, aged animals often reside in the same lab for most of their lives and have undergone considerable cognitive testing, which could enhance their cognitive performance, when compared to experimentally naïve aged animals. Cognitive performance of aged dogs also is also enhanced by prior cognitive test experience (Milgram, 2003; Ikeda-Douglas, et al., 2003).

The present study sought to further extend the canine model of cognitive aging with a 2-year longitudinal investigation of discrimination and reversal learning ability in groups of young and

aged beagle dogs. We had two major goals. The first was to obtain longitudinal data of age-dependent decline in learning ability. The second was to assess the effectiveness of two interventions in counteracting age-dependent decline, behavioral enrichment and maintenance on a food supplemented with a broad spectrum of antioxidants and mitochondrial cofactors.

1.1 Behavioral Enrichment and Age Dependent Cognitive Decline

The behavioral enrichment intervention included three components: increased exercise, environmental enrichment, and possibly most important, a program of cognitive enrichment. Exercise was suggested primarily by studies indicating that physical activity is associated with improved cognitive function and lower risks of cognitive impairment and dementia (e.g., Cotman & Berchtold, 2002; Churchill et al. 2002). The second component, environmental enrichment was suggested by evidence that rearing in enriched environments improves learning ability, produces beneficial changes in cellular structure and increases the résistance of neurons to injury (Mattson et. al. 2001). The effect can be sufficiently robust as to reduce or eliminate age-dependent cognitive decline (e.g., Escorihuela, Tobena & Fernández-Teruel,1995).

The rationale for including cognitive enrichment intervention was based on retrospective studies of human subjects suggesting a link between cognitive experience and the development of age-dependent cognitive dysfunction. People characterized as having a low-level of cognitive function are more likely to develop severe cognitive dysfunction than people characterized as having a high level of cognitive function (Greiner, Snowdon, Schmitt, 1966). Similarly, several studies have reported an inverse relationship between amount of education and rate of cognitive decline later in life. More direct evidence has been obtained in studies demonstrating that special training protocols improve cognitive performance in the elderly (Ball et. al., 2002), and in patients with dementia (Moore et al., 2001).

1.2 Antioxidant supplementation and age-dependent cognitive decline

The dietary intervention consisted of providing a specially formulated canine food containing a broad spectrum of antioxidants and mitochondrial cofactors. The food was intended to reduce damage to tissue by reactive oxygen species. Reactive oxygen species are formed as by-products of cellular metabolism and, when produced in amounts in excess of detoxification, are purported to cause oxidative stress (Beckman and Ames 1998). Oxidative damage to proteins and lipids has been linked to the development and accumulation of neuropathology associated with degenerative disease as well as normal aging (Aksenova et al.,1999; Gabbita et al., 1998; Markesbery and Lovell, 1998; Miranda et al., 2000), and is therefore a likely causal factor in age-dependent cognitive decline.

There is also more direct evidence of beneficial effects on cognition of dietary supplementation with antioxidants. In aged rats, antioxidants improved spatial learning (Socci et al.,1995, Joseph et al., 1998), motor learning and cerebellar function (Bickford et al., 2000; Cartford et al., 2002). The effectiveness of antioxidants in counteracting age-related cognitive decline has also recently been demonstrated in mice deficient in apolipoprotein (Veinbergs et al., 2000), vitamin E deficient aged rats (Fukui et al., 2002), and in the SAMP8 mouse (Farr et al., 2003). In clinical trials, antioxidant supplementation of Alzheimer's patients with vitamin E was found to delay the onset of institutionalization (Sano et al., 1997). We have also found that short-term maintenance on an antioxidant fortified food improved discrimination learning in beagle dogs (Milgram et al., 2002a; Milgram Zicker et al., 2002b). Furthermore, we have

previously shown evidence of increased oxidative stress in the aged canine brain (Head et al., 2002).

The present investigation started with a period of baseline testing, which was used to separate beagle dogs, approximately 8 to 11 years of age, into four cognitively equivalent groups, which differed in food provided and behavioral enrichment. We hypothesized that both the behavioral and dietary enrichment treatments would have beneficial effects on cognitive function, and that the two treatments combined would be more effective than either separately. To partially evaluate this hypothesis, one year after the treatment phase was initiated the animals were tested successively on a size discrimination learning task and a size discrimination reversal learning task. These tasks were selected because they were conceptually similar to the object discrimination learning and object discrimination reversal tasks used in assessing baseline cognitive function. Furthermore, we have previously found the size and size reversal tasks to show age sensitivity (Head et al 1998., Tapp et al. 2003). The one year results been previously reported (Milgram et al.,2003 in submission). Both the fortified food and behavioral enrichment improved learning in both tasks, but this was mainly due to the combined treatment group (fortified food and enriched environment) showing the greatest improvement. Approximately two years following the start of the treatment phase, the animals were tested on a black/white discrimination and a black/white discrimination reversal learning task, to provide a protocol for assessing longitudinal changes in discrimination and reversal learning and the effectiveness of the fortified food and behavioral enrichment interventions over two years.

2 Materials and Methods

2.1. General Design

Forty-eight old and 16 young beagle dogs were trained on a battery of cognitive tests over approximately 9 months. Performance on the baseline testing was then used to divide the aged dogs into four cognitively equivalent test groups of 12 animals each, with two treatment conditions – dietary supplement and behavioral enrichment. Group, C-C, was both fed the control food and provided with control enrichment; Group, C-E, received the control food and a program of behavioral enrichment. Group A-C dogs were fed food fortified with antioxidants and mitochondrial cofactors and also given control enrichment: while Group A-E received both the fortified food and the behavioral enrichment

The young beagle dogs were divided into two cognitively equivalent groups, one of which was given the antioxidant enriched food (N=9) and the other the control food (N=7). One control dog was subsequently dropped from the study during the first treatment year because of motivational problems, reducing the size of the control group to 7. Both young groups received the behavioral enrichment protocol.

One year after the start of the dietary manipulation, all dogs were tested on both a size discrimination and reversal learning task. Prior to the one-year test, dogs in the enriched environment groups had participated in a landmark discrimination task (Milgram et al., 2002) and an oddity discrimination task (Milgram et al. 2002b). The size discrimination task evaluated ability to learn to distinguish two objects that differed only in size in order to locate a food reward. In the size discrimination reversal task, the association between a particular object and reward was switched. Thus, if an animal was rewarded for approaching the smaller of two objects during the initial discrimination learning task, it was rewarded for approaching the larger

of the two objects during the reversal task. The size discrimination results have been previously reported (Milgram et al., 2003).

The enrichment condition between years 1 and 2 of treatment consisted of training on a size concept learning task (e.g., Tapp et al., 2003) and on a repeated reversal learning task. At the completion of the the 2 year enrichment phase, all animals were trained on a black/white discrimination learning task in which the animals were presented with two blocks that were identical in size and shape but differed in color, with one object painted white and the other black. Animals were first trained to approach one of the two to obtain a food reward. After achieving a criterion level of performance, the rewarded objects were then switched during the black/white discrimination reversal phase.

2.2 Subjects

Two groups of beagle dogs (Canis familiaris) served as subjects. The first consisted of 48 aged canines (24 males and 24 females) ranging from 7.7 to 11.6 years at the start of baseline testing, and from 9.3 to 13.3 years of age at the start of the treatment phase. The second group consisted of 17 young canines 13.8 years of age at the start of testing (6 males and 11 females). Half the dogs (young and old) came from a closed colony of beagle dogs (Cohort 1). The other half were obtained from a second, independent, closed colony (Cohort 2).

The aged canines were housed, either singly or in pairs, in pens with continual access to fresh water. Because of space considerations, the young canines were housed in a separate animal facility, from 2 to 4 per room. In all other respects, the old and young animals were treated identically. All dogs were fed approximately 300g of the control food once daily.

. All animals were administered a full physical and neurological examinations prior to dietary intervention and again at 6 and 12months after the start of intervention. Dogs were also examined by slit-lamp for ocular abnormalities that might have impaired the animals visual capabilities. The physical examination did not reveal any neurological, musculoskeletal, ocular or physical abnormalities that justified exclusion from the study.

2.3 Apparatus:

The test apparatus was a .609-m x 1.15-m x 1.08-m wooden chamber that was based on a canine adaptation of the Wisconsin General Test Apparatus (see Milgram et al. 1994) The testing chamber was equipped with a sliding Plexiglas food tray with three food wells. Vertical stainless steel bars covered the front of the box. The height of the bars was adjustable to allow the size of the opening to each food well to be uniquely adjusted for each canine. The experimenter was separated visually from the dog, by a wooden screen containing a one-way mirror, and a hinged wooden door at the bottom. Testing was conducted in darkness, except for a light with a 60-watt bulb attached to the front of the box. Each test trial commenced with the hinged door being opened for the presentation of the tray. A one cm^3 amount of Hill's Prescription Diet Canine p/d was used as the food reward.

2.4 Dietary Intervention

The control and antioxidant foods were formulated to meet the nutrient profile for the American Association of Feed Control Officials recommendations for adult dogs (AAFCO 1999). The two foods were identical, except for the inclusion of a broad-based antioxidant and mitochondrial cofactor supplementation to the test food. The control and enriched foods had the following differences in formulation on an as fed basis respectively: dl-alpha-tocopherol acetate, (120 ppm

vs 1050 ppm), l-carnitine (< 20 ppm vs 260 ppm), dl-alpha-lipoic acid (<20 ppm vs 128 ppm), ascorbic acid as Stay-C (< 30 ppm vs 80 ppm), and 1% inclusions of each of the following (1 to 1 exchange for corn): spinach flakes, tomato pomace, grape pomace, carrot granules and citrus pulp. The rationale for these inclusions is as follows: Vitamin E is lipid soluble and acts to protect cell membranes from oxidative damage; vitamin C is essential in maintaining oxidative protection for the soluble phase of cells as well as preventing vitamin E from propagating free radical production; alpha-lipoic acid is a cofactor for the mitochondrial respiratory chain enzymes, pyruvate and alpha-ketoglutarate dehydrogenases, as well as an antioxidant capable of redox recycling other antioxidants and raising intracellular glutathione levels; L-carnitine is a precursor to acetyl-l-carnitine and is involved in mitochondrial lipid metabolism and maintaining efficient function; fruits and vegetables are rich in flavonoids and carotenoids and other antioxidants. The diet was produced by an extrusion process and was fed for no more than 6 months before a new lot was manufactured.

2.5 Behavioral Enrichment Intervention

The behavioral enrichment condition commenced after completion of the baseline testing period. The animals in the enriched group were housed with kennel mates, exercised twice a week for 15 minutes intervals, and given sets of toys that were alternated weekly. None of these were provided to the control animals. The enrichment condition also included a cognitive enrichment protocol. The year first year of cognitive enrichment started immediately after baseline with testing on a series of landmark discrimination problems, as previously described (Milgram et al., 2002). After completing the landmark task, the subjects were tested on to a series of oddity discrimination leaning problems (Milgram Zicker et al., 2002). After completing the oddity problems, the dogs were then tested for retention of the landmark task. The cognitive enrichment during the second year consisted of a series of nine discrimination learning tasks that were intended to study size concept learning (Tapp et al., In submission , and a series of repeated reversal learning tasks that were intended to study learning set formation.

2.6 Cognitive Test Protocol

2.6.1 Pretraining

All subjects underwent a standard pretraining cognitive testing protocol that consisted of reward approach and object approach learning (Milgram et al., 1994), which were procedural learning tasks designed to train animals to displace an object on a tray to obtain a food reward consisting of approximately 1 gm of Hill's Prescription Diet® Canine p/d canned food. This food served as an effective reward for all of the dogs used in the study. After completing the procedural learning tasks, all subjects were trained on an object discrimination learning task, which was followed by an object reversal learning task (Milgram et al., 1994). The animals were then tested on an object recognition memory task (Callahan et al., 1999) and delayed-non-matching-to-position task (DNMP) (Chan et al., 2001). The initial group assignment took into consideration age, sex, cohort and the subjects combined performance on the reversal learning task, the object recognition task, and the DNMP task. The analysis of the baseline cognitive data has previously been reported (Milgram et al., 2002). The four test groups of aged dogs did not differ on any of the baseline tests used in classification. Similarly, the two test groups of young animals did differ from each other on the baseline evaluations. There were, however, significant differences between the old and young animals in performance on the reversal and visuospatial tasks. All

animals were maintained on the control food during the pretraining period, which continued for approximately 9 months.

2.6.2 Year 1 assessment

Testing on the size discrimination learning task commenced approximately 20 months following the start of baseline testing, and approximately one year after the start of the treatment phase. For the animals in the cognitively enriched group (Groups CE and AE), a one-week non-test interval preceded the start of size discrimination learning. The dogs in the control environmental (AC, CC) condition did not undergo any cognitive testing for approximately 9 months after completing the baseline testing. The procedure followed in the size and size reversal learning has been described previously (Milgram et al., 2002; Tapp et al., 2002).

2.6.3 Year 2 assessment

The black/white intensity discrimination task training started approximately two years after starting the treatment conditionst. We used two wooden blockswhich were identical in all respects except color: one was covered with a white enamel paint and the other a black enamel paint. The training procedure started with a preference test, which consisted of a single test session used to establish object preferences; the total choices of one the blocks out of 10 provided the absolute preference score. On this and all subsequent test sessions, the objects were placed over the two lateral food wells, and the location of the objects varied randomly, with the constraint that each object was placed on each side on 50% of the trials. A customized computer program controlled all timing and randomization procedures. The program also assured that on each trial, the locations of the objects were the same for each animal. Before the beginning of each trial, the computer emitted a tone that served as a cue for the dog and instructed the experimenter to present the food tray. Each trial was started when the experimenter pressed a key and simultaneously presented the tray to the subject. The dogs' responses were recorded by a key press, which also indicated the end of the trial and signalled the beginning of the inter-trial interval.

Training on the black/white intensity discrimination problem started on the day following the preference test. The animals were given 10 trials per day, with an intertrial interval of 30 seconds. Testing was 6 days per week. The animals received a maximum of 40 training sessions to achieve a two-stage criterion. The first stage was successfully met once the animal either averaged 80% over two sessions, or at least 90% on a single session. To complete the second stage, the dog was required to respond correctly on at least 70% of the trials over three successive trials. Thus, passing both stages took a minimum of four test sessions. One dog failed to learn the black/white discrimination task within the 40 trials, and was consequently administered a program of remedial training, so that the dog could be tested on the reversal task. During the remedial training phase, an additional 13 training sessions were allotted for each animal to reach the criterion performance level.

The black/white reversal task started on the day following completion of the initial discrimination learning task. The testing procedures were identical, except that the reward contingencies of the positive and negative block were reversed. Thus, if an animal was rewarded for approaching the white block during the initial testing, it was now rewarded for approaching the black block.

2.7 Statistics

For individual subjects, rate of learning was characterized by error scores, which were calculated by adding the total number of errors to either pass the two-stage learning criterion, or the total

number of errors made over 40 training trials. The data were analyzed with factorial and repeated measures analysis of variance (ANOVA's). When required, Post-Hoc analysis was performed by Tukey's Stdentized Range Test (HSD), using the 0.05 level of significance. In addition, chi square analysis was performed on the frequency of failure for the three assessment periods.

3 Results

3.1 Survival

Table 1 shows the sample size and mean ages of each group at the start of discrimination testing during baseline, after one year of treatment and after two years. As indicated in Table 1, completed sets of discrimination and reversal data were not obtained from 4 of the animals assigned to group C-C, and 1 animals assigned to group CE: Four of these died or had to euthanized for medical reasons; the fifth was dropped from the study because of motivational problems.

3.2 Effect of Food and Experience on Learning a Black/White Discrimination and Reversal Task

For the aged animals, the results of the black/white task were first analyzed with a repeated measures analysis of variance, with discrimination and reversal learning as within subject measures, and source, food, and behavioral enrichment as between subject measures. The results revealed significant main effects of food ($F(1,34)=4.678$; $p<0.05$), behavioral enrichment ($F(1,34)=31.89$, $p<0.01$) and task ($F(1,34)=78.93$; $P<01$). As expected, the task effect was due to the reversal task being more difficult than the original discrimination learning task.

To further breakdown the food and behavioral enrichment effects, we performed separate factorial analyses for the black/white discrimination task and for the black/white discrimination reversal task. On the black/white discrimination task, the ANOVA revealed a significant main effect of behavioral enrichment ($F(1,38)=22.35$; $p<0.01$), and no other significant effects or interactions. Figure 1a illustrates that each of the groups that received behavioral enrichment (Group AE and Group CE) made fewer errors than either of the two non-enriched groups (Group AC and Group CC). Tukey's LSDs indicated that each behavioral enrichment groups performed significantly better thanits respective control enrichment group (Group CC vs CE and Group AC vsAE). By contrast, the control groups did not differfrom each other.s differ from each other.

Analysis of black/white discrimination reversal learning, on the other hand, revealed significant effects of both behavioral enrichment ($F(1,34)=27.2094$; $p<0.01$) and food ($F(1,34)=5.11$; $p<0.05$). These results are largely due to the high level of performance of group AE (see Figure 1B), which received the combined treatment of antioxidant enriched food and behavioral enrichment. Further, post hoc analysis revealed that group AE did significantly better than group CC and group AC. The only other significant group differences were between group CE and group CC.

We also analyzed the data from the young animals using a repeated measures ANOVA, and found a significant effect of task, but no other significant main effects or interactions (See Figure 2).

3.3 Age differences

Because the young animals all received behavioral enrichment, age differences were assessed with the use of a repeated measures ANOVA that compared the two young groups with the old groups provided with behavioral enrichment. The results revealed a highly significant effect of

task (F(1,34)=48.93; p<0.01), age (F(1,34)=48.93; p<0.01), and a significant age by task interaction. (F(1,34)=5.799; p=0.021). To further clarify results separate factorial analysis were carried out for black/white discrimination and black/white discrimination reversal learning.

There were no significant differences in the discrimination learning task. On the reversal task, by contrast, there was a highly significant age effect (F(1,34)=17.12; p<0.01). As indicated in Figure 3, this was largely due to poor performance of the old animals on the control food. Post-hoc analysis indicated this group performed significantly more poorly than the old and young animals on the enriched food. The difference between the old animals on the control food and the young group on the control food was marginally significant (p= 0.068).

3.4. Longitudinal changes in discrimination and reversal learning between baseline and year 2 assessment

3.4.1 Overall results with all animals, young and old included

To evaluate longitudinal changes in discrimination and reversal learning, the data over the three years were first analyzed with an omnibus repeated measures ANOVA with age (young vs old) and food (enriched vs control) as between subject variables and test year (object, size and intensity) and task type (discrimination vs reversal) as within subject factors. There were highly significant main effects of age (F(1,54)=44.277; **p=0.00000**) and test year (F(2,108)=21.149) task type (F(1,54)=273.67; **p=0.00000**). There were also interactions between age and test year (F(2,108)=10.446; p=0.00071) and age and task (F(1,54)=24.76; p=0.000007).

3.4.2 Effects of age within behaviorally enriched groups

To examine the effects of age, we next performed the same analysis on the behaviorally enriched dogs only, which included all of the young dogs and half of the old dogs. The results revealed a highly significant effects of food (F(1,34) = 7.22; p=0.011093), test year (F(2,68)=15.34; p=0.000003) and test type (F(1,34)= 142.04; **p=0.00000**). There were also significant interactions between age and test year (F(2,68)=7.75; p=0.000928) and between age and test type (P(1,34)=18.9153). As illustrated in Figure 4, the task and age effects are attributable to animals generally showing faster learning of the discrimination task than of the reversal task, and second, consistently more accurate learning by the young animals. *3.4.3 Effects within aged animals only*

We then looked at treatment effects in the aged animals alone, looking at discrimination and discrimination reversal learning separately. For the discrimination learning task, there were highly significant main effects of task (F(2,68)=34.11, **p=.0000**) and experience (F(1,34)=17.95, p=.00016) and significant two way interactions between task and experience (F(2,66)=7.475, p=0.001) and between task and food (F(2,66)=34.11, p=.0037). Figure 5 shows that the task effect reflects the animals showing a progressive slowing in learning. The interaction with experience reflects the behaviorally enriched group performing better than the non-enriched group over the third year only. Finally, the task by food effect reflects consistently better performance of the enriched animals over the controls on the last two years, after the start of the antioxidant treatment (Figure 5B appears to have legends reversed).

The reversal learning was first analyzed with a repeated measure ANOVA over the three years and revealed significant main effects of enrichment (F(1,34)=9.78 p=0.00036), food (F(1,34)=4.198, p=0.0482), and task (F(2,68)=52.11, p=0.000). There were also significant interactions between task and enrichment (F(2,68)=15.60, p=0.000) and between task and source (F(2,68=3.37) p=0.04) . Figure 5 (bottom) illustrates that the food effect is largely due to the

performance of the group given both the antioxidant enriched food and the behavioral enrichment over the first and second year of the study. The behavioral enrichment effect is shown in Figure 5 (top), which illustrates that the animals provided with the enriched treatment learned more accurately than the animals provided with control enrichment, and this increased over repeated testing.

The subjects tested in these experiments were allowed a maximum of 40 sessions to solve the reversal learning task and some animals were unsuccessful. The error score assigned to the subjects that failed was based on the 40 test sessions administered, which underestimated the true error rate because of a ceiling effect. The number of failures for each of the test groups is shown in Table 2. The contrast between the animals given the combined treatment and the animals in the control-control group was notable. All 12 of the animals in the combined treatment condition were able to solve all of the reversal learning problems, while only 2 of 8 control-control animals showed learning. The chi squared value obtained by comparing frequency of failure for the discrimination reversal learning over all time periods with expected frequencies was highly significant ($p=0.0112$). Subsequent chi-square analysis of the failure at each measured point showed no significant difference at baseline or the first year of assessment. However, the second year of assessment revealed a highly significant chi-square of 0.0033. This could be attributed to the high failure rate in the CC group compared to the other groups.

Because of the large contribution of the baseline data, we also performed a repeated measures ANOVA over the last two years only. The results again yielded a significant effect of task ($F(1,39)=386.48$, **$p=.0000$**). We also found a significant effect of source ($F(2,68)=34.11$, **$p=.0000$**), a significant interaction between source and task ($F(1,39)=6.17$, $p=.0017$) and a significant three way interaction between experience, food and task ($F(1,39)=4.40$, $p=.042$) These results were strongly influenced by the differences in difficulty between the baseline object discrimination task and the tasks used in follow-up assessments at year 1 and 2. To examine the effects of behavioral enrichment and food, independently of the baseline data, the results from the aged subjects were analyzed using a repeated measures ANOVA with size discrimination and intensity discrimination as repeated factors and food, experience and source as dependent variables. The results of the analysis revealed significant main effects of food ($F(1,34) = 4.87$, $p=0.034$), and enrichment ($F(1,34)= 16.23$, $p= 0.00003$), and a significant interaction between task and enrichment ($F(1,34)=9.19$, $p=0.004$).

4 Discussion

This project had three goals: to evaluate the cognitive effectiveness of long-term maintenance on an antioxidant-fortified food; to evaluate the effectives of a long-term program of behavioral enrichment, and; to assess cognitive decline in the beagle dog in a longitudinal study. The data presented in this report was obtained from aged dogs tested on a black/white discrimination learning and reversal task, after two years receiving one of four treatment conditions: control food, control enrichment; fortified food – control enrichment, control food – behavioral enrichment and fortified food – behavioral enrichment. We also tested two groups of dogs that were young at the start of the study. One group received the control food and the other the fortified food. Both groups of young dogs received behavioral enrichment.

In general, both treatment conditions improved performance of the aged group, but not the young animals. However, the effectiveness of the various treatment combinations varied as a function of both task and age. Performance of the black/white discrimination learning task was significantly improved in the aged dogs provided with behavioral enrichment, relative to the control enrichment condition. The reversal task, by contrast, was significantly affected by both task and food. Furthermore, because the experimental design limited the number of the training trials, the magnitude of the treatment effects was likely underestimated. Eight of 18 animals in the control food condition failed the reversal test in the allotted 40 sessions and 10 of 20 animals in the control environment failed. By contrast, all 12 animals in the combined treatment condition successfully achieved the *a priori* learning criterion. Finally, on both tasks, the two treatments combined were more effective than either treatment alone.

The performance of the subjects in young group by contrast, was unaffected by the dietary manipulation, which was not surprising. The effectiveness of the food is theoretically linked to it's ability to arrest or reverse cellular dysfunction produced by excessive free radicals. However, free-radical based brain damage is minimal in younger animals (Heat et al.,).

The results of this study extend our previous report on the effects of the food and behavioral enrichment on size discrimination and reversal learning, which was carried out after one year of treatment. Whereas the present study revealed a significant main effect of food by itself on the reversal learning, the one-year results indicated that antioxidant supplementation was only effective when it was combined with behavioral enrichment (Milgram et al., 2003). The present results indicate that the effect of enriched food on cognition is more robust after 2 years on the food than it is after only one year. We have also found, however, that dietary fortification has significant beneficial effects after a short time frame among animals provided with behavioral enrichment (Milgram et al., Milgram et al.,)

4.1 Effects of behavioral enrichment

The behavioral enrichment condition included a program of cognitive enrichment, increased physical activity and environmental enrichment. The present results do not allow us to distinguish the relative importance ofn each of these interventions. We suspect, however, that the cognitive enrichment was particularly important. First, previous animal studies in which aged subjects are provided with environmental enrichment have had small inconsistent effects on cognition (van Gool et al. (1985). By contrast, training animals on particular tasks early in life can produce long-lasting changes in the animals abilities to learn those tasks later in life (e.g., Vicens et al., 2002).

Cognitive enrichment protocols have also been found to produce beneficial effects in elderly human subjects. Ball et al. (2002) examined the effect of 3 distinct cognitive interventions (memory training, reasoning training, and speed of processing training). They were all effective, but the effectiveness was selective, and improved only the targeted cognitive ability. These results from human subjects generally suggest that cognitive enrichment protocols have effects that are task specific. The present results are consistent with this conclusion. The cognitive enrichment protocol consisted of a broad range of tasks that could be solved with a discrimination learning strategy. This was also true of the black/white discrimination task, and the discrimination reversal task, which requires two cognitive skills: inhibiting the response to a previously rewarded stimulus and learning to respond to a previously non-rewarded stimulus. .

4.2 Effects of dietary intervention

The observation of improved performance in the groups on the antioxidant fortified food is consistent with our previous reports, which were obtained in animals provided with the enriched food for under a year. The present results extend these findings to indicate that the enriched food remains an effective therapeutic after two years of treatment. The dietary intervention used in this study has been previously described and discussed (Milgram et al., 2001; Milgram et al., 2002; Cotman et al., etc.) . Briefly, the food was enriched with a cocktail containing both antioxidants and mitochondrial cofactors. Because of the numerous ingredients, we do not know which if any of the components are most important, or, alternatively, whether beneficial effects depend on the use of a broad spectrum of ingredients. The latter interpretation is consistent with the moderately large literature on the effects of antioxidant supplementation on cognition. In several studies, in which only a limited group of antioxidants were used, the effects on cognition were small and restricted. Thus, Sano et al., (1977) found that supplementation with α-tocopherol for a period of two years produced significant changes in functional measures but did not improve scores on cognitive measures such as on the mini-mental state examinations in moderately demented Alzheimer's disease patients (Sano et al., 1997). Socci et al. (1995) found no effect on passive avoidance memory of long-term antioxidant supplementation with vitamin E., phenyl-α-tert-butylnitrone and ascorbic acid, although the antioxidant treatment did improve rate of water maze learning. Finally, Joseph et al. (1999) did not find differences between control treatment and a variety of antioxidants on water maze learning, although they reported evidence suggesting that supplements resulted in greater improvement, and also were able to erase other age-dependent deficits.

4.3 Cross sectional age differences

The analysis of age dependent cognitive decline was partially confounded by the absence of a non-enriched young-animal group. The young animals, consequently, could only be compared with the behaviorally enriched aged-animals. The results revealed significant differences between the age-groups on the reversal learning, but not on the discrimination learning. The absence of an age-dependent difference in discrimination learning contrasts with data obtained from these animals after only one year of treatment (Milgram et al., 2003), and with other studies showing age-dependent deficits in complex discrimination learning tasks (e.g., Tapp et al.,). We attribute these results to two factors: the first is the extensive test experience given to our aged animals. The second relates to the age-range of the young group. Although we've used the term young, the mean age of the young group at the time of final testing was greater than 5 and some of the animals were over 7, which would actually characterize these animals as middle aged (Milgram et al., 1994).

We did get significant age differences in the reversal learning task, as expected based on our previous work (Milgram et al., Tapp et al., Milgram et al.,) and studies with non-human primates (Bartus et al., ; Lai et al., 1995; Voyto., Rapp). However, the differences were largely a result of poorer performance of the aged group that received the control food, suggesting that the antioxidant food was able to reduce age-dependent deficits in reversal learning.

4.4. Longitudinal changes in cognitive ability in the young and old groups

The other aim of this study was to obtain longitudinal evidence of changes in discrimination and reversal learning ability in the beagle dog. To characterize cognitive decline, we studied two groups of beagle dogs, an aged group and a young group. The aged group consisted of 48 dogs

that ranged from approximately 8 to 10 years of age. The young group consisted of 16 dogs. The results demonstrated progressive deterioration in performance over three years in both discrimination and reversal learning in the aged dogs.

At the start of the study, the old group performed significantly worse than the young on object discrimination reversal learning, but not on object discrimination learning, suggesting overall cognitive impairment in 9 to 11 year old beagle dogs. As a group, the aged animals showed progressively poorer performance throughout the course of the study on both discrimination and reversal tasks. Thus, their performance on size discrimination and reversal learning was significantly better than their performance on the black/white discrimination. Conclusions about age differences are confounded by possible differences in both task difficulty and in the importance of previous test experience. Nevertheless, the performance differences between the object and black/white tasks are likely due to marked age-dependent deterioration over the two-year period, particularly in the control groups. This suggestion is supported by a previous experiment, in which a crossover design used to compare acquisition of a size discrimination and black/white discrimination tasks, indicated that the size task was significantly more difficult than the black/white task for aged beagle dogs (Milgram Siwak et al., 2000). In the present study, by contrast, the aged dogs did more poorly on the black/white discrimination, which was tested in the second year of the study, than they did on the size discrimination task, which was tested in the first year of the study. Since this is the opposite of what we've seen previously, it's likely that the poorer performance reflects age differences (the animals were one year older) rather than differences in task difficulty. This conclusion is further reinforced by the data from the young dogs, which performed comparably on the black/white,size and object discrimination and also on the discrimination reversal tasks.

Another notable observation was the high incidence of failures – particularly by the control group on the reversal learning task. Because these tasks are not particularly difficult for young or middle aged beagle dogs, learning failures provide strong evidence of age-associated cognitive decline or dementia. The present results, therefore, suggest that the likelihood of cognitive decline increases precipitously after the age of 10. This conclusion is also consistent with the results of Patronek et. al. (1997), in which a 10 year old beagle was found to be equivalent in physiological age to a 66.6 year old human. We have also found evidence of precipitous cognitive deterioration in the performance of dogs used in this study on a delayed non-matching to position task, a measure of visuospatial function, which will be reported separately. Several aged dogs failed the task in the third year of the study, despite the fact that they had successfully learned the task when tested one or two years earlier (Milgram et al., In Preparation).

We have focused primarily on the old dogs. There was also a suggestion, however, of cognitive deterioration in the young dogs in the last year of the study.The performance of the young group showed an overall decline in the second year of the study, when they were tested on the black/white discrimination. However, we expected the dogs to do better on the black/white test than on the size discrimination based on the findings of Milgram, Siwak et al.,(2000). In fact, by the second treatment year of the study, the designation of the group as young dogs was no longer appropriate, as the mean age of the dogs was now over 5 years. One possibility is that the performance on the black/white discrimination task actually represents impaired performance, relative to younger dogs.

Collectively, these results suggest a possible time frame for the development of cognitive deterioration in the beagle dog. Based on the young dog data, the results suggest that maximal

performance is typically reached between about 2 and 4 years of age, and that performance begins to fall off starting around 5 years of age. By 8 years of age, there are clear and consistent age-dependent impairments, which are likely to relate to frontal lobe dysfunction. Subsequently, cognitive decline increases at an accelerated, and after about 12, an increasing proportion of animals show severe decline and can characterized as demented.

This suggested time frame for cognitive aging in the beagle dog is consistent with imaging studies of aged dogs, and time course studies of beta amyloid deposition. Su et al. (1998) found that after the age of 10, ventricular volume increases exponentially. Head et al. (2000) found that neuropathogy, manifested by the occurrence of beta amyloid deposition, first appears when the beagle dog is about 8, and at that time is largely restricted to the prefrontal cortex. At this time, many cognitive functions are unimpaired, although deficits are manifested in functions such as reversal learning, that are likely frontal lobe dependent. After 10 years of age, beta amyloid is also notable in entorhinal, parietal, and occipital cortex, which is also the time frame when more severe and widespread cognitive dysfunction occurs.

The suggestion that of an increasing proportion of subjects with severe cognitive decline after about 12 is also consistent with observational data obtained from studies of pet dogs, in which cognitive impairment characterized by disorientation, disturbances in social interactions, impairment in house training and disruption of sleep-wake cycles shows an increasing prevalence with advanced age. Thirty percent of 11- 12 year-old animals show impairment in one or more category and 10 percent show impairments in two or more of these categories. In animals between 15 and 16, the percentages increase to 68% and 35% (Neilson et al., 2001).

4.5 Conclusions

To conclude, the present results demonstrates that discrimination and reversal learning ability declines progressively with advanced age in beagle dogs, but that the rate of decline can be delayed by both behavioral and antioxidant supplementation. Possibly the most important outcome of this study was the demonstration that the behavioral enrichment and the antioxidant supplementation condition combined were more effective than either alone. This study is the first that we know of to look at both interventions in combination. The dietary intervention was based on a cocktail of compounds, and it will be important in future studies to determine which of the ingredients are most effective, and whether the cocktail can be improved. The behavioral intervention also involved a cocktail of treatments (activity, environmental enrichment and cognitive enrichment), and the contribution of each to the present results remains to be determined.

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Table 1: Mean Age of Groups at Start of Discrimination Testing over Three Years

Age Group	Object Discrimination (Baseline)		Size Discrimination (Year 1)		Intensity Discrimination (Year 2)	
Old	N	Age (Years	N	Age(Years)	N	Age(Years)
C-C	12	9.95 + 1.01	12	11.66 ± 0.97	8	12.87 ± 0.89
C-E	12	9.98 + 0.93	11	11.48 ± 0.89	11 (10 in table 2)	12.58 ± 0.88
A-C	12	9.70 + 1.24	12	11.04 ± 1.64	12	12.50 ± 1.26
A-E	12	9.45 + 1.15	12	11.15 ± 1.13	12	12.24 ± 1.17
Young						
A	9	2.58 ± 1.08	9	4.26 + 1.13	9	5.41 + 1.15
C	7 (8 in paper)	2.39 ± 1.08	7	3.69 + 1.09	7	5.06 + 1.11

Mean ages and and standard deviations of the groups at the start of discrimination learning testing at baseline, after one year of treatment, and after two years.

Treatment Group	Year 1- Object Reversal	Year-2 Size Reversal	Year-3 Black/White Reversal
Control Control	0/12	3/12	6/8
Control Enriched	0/12	3/12	2/10
Antioxidant Control	0/12	3/12	4/12
Antioxidant Enriched	0/12	0/12	0/12
Total	0/48	9/48	12/42

Table 2. Reversal Learning Failures as a Function of Task and Treatment Group

Figure Captions:

Figure 1. Effect of antioxidant enriched food and behavioral enrichment in on acquisition of a black/white discrimination learning task (top) and reversal learning task (bottom) in aged beagle dogs. Error bars represent standard errors.

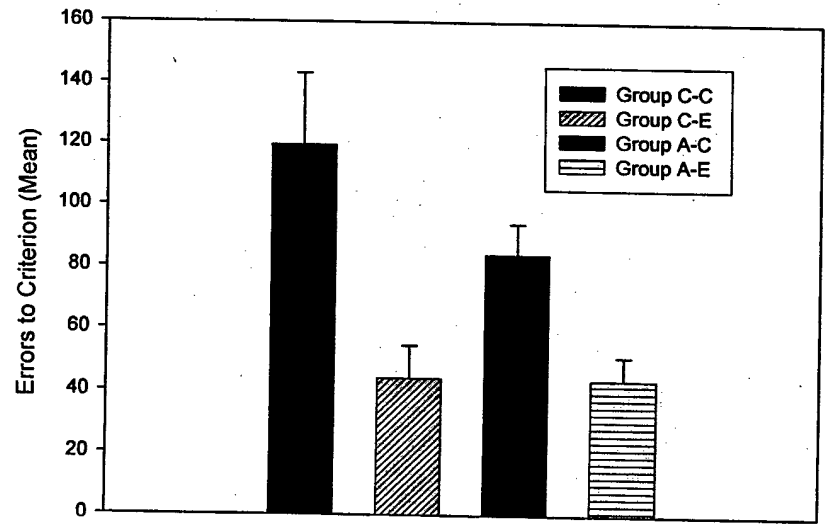
Figure 2. Effect of antioxidant enriched food on acquisition of a black/white discrimination and black/white discrimination reversal learning task in young beagle dogs. Error bars represent standard errors

Figure 3. Age differences in acquisition of black/white discrimination and black/white discrimination reversal learning task. The old animal data was taken only from subjects in the behaviorally enriched groups. Error bars represent standard errors.

Figure 4. Longitudinal changes in discrimination (top) and reversal learning (bottom) in young and old beagle dogs. The aged group included in the figure were limited to the subjects in the behaviorally enriched groups.

Figure 5. Performance on Discrimination Learning Tasks as a Function of Food and Behavioral Enrichment. Top shows scores over three years for the animals in the behaviorally enriched and control enrichment groups. Note the absence of any differences until year 3. The bottom compares the aged animals on the antioxidant fortified and control foods at baseline and over the next two years.

A



B

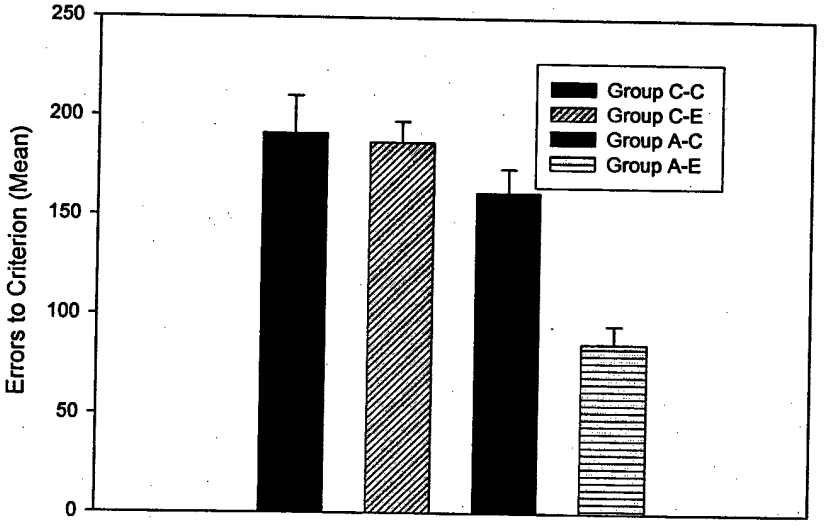


Figure 1 (Aug 4, 2003)

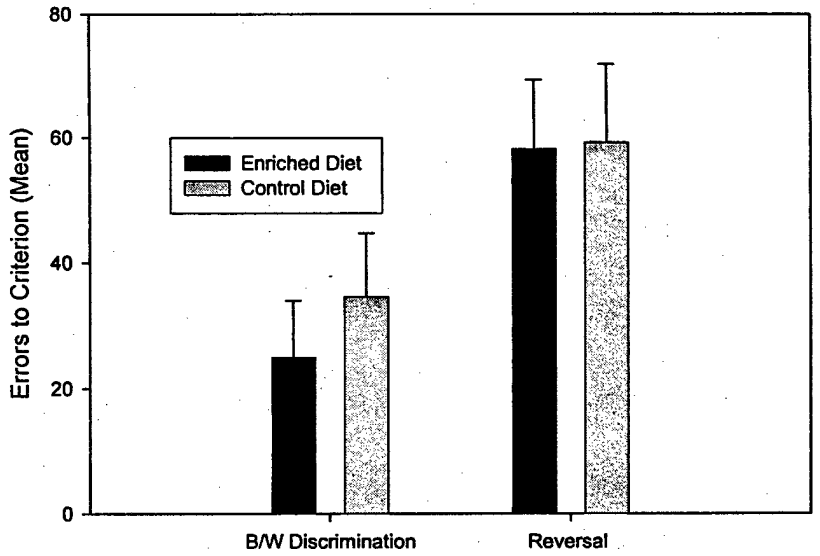


Figure 2 – Effect of food on Black/White discrimination And reversal learning in young animal group.

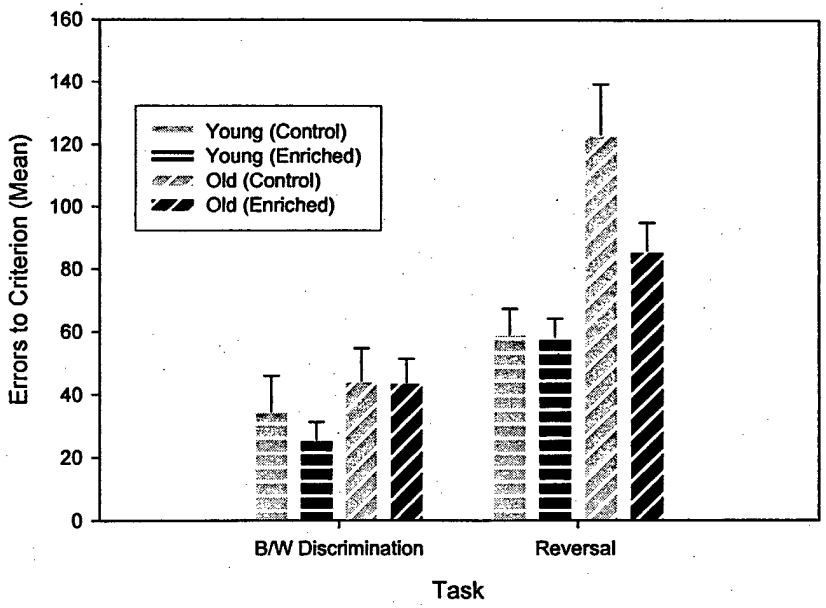


Figure 3. Age differences in acquisition of B/W discrimination and reversal learning.

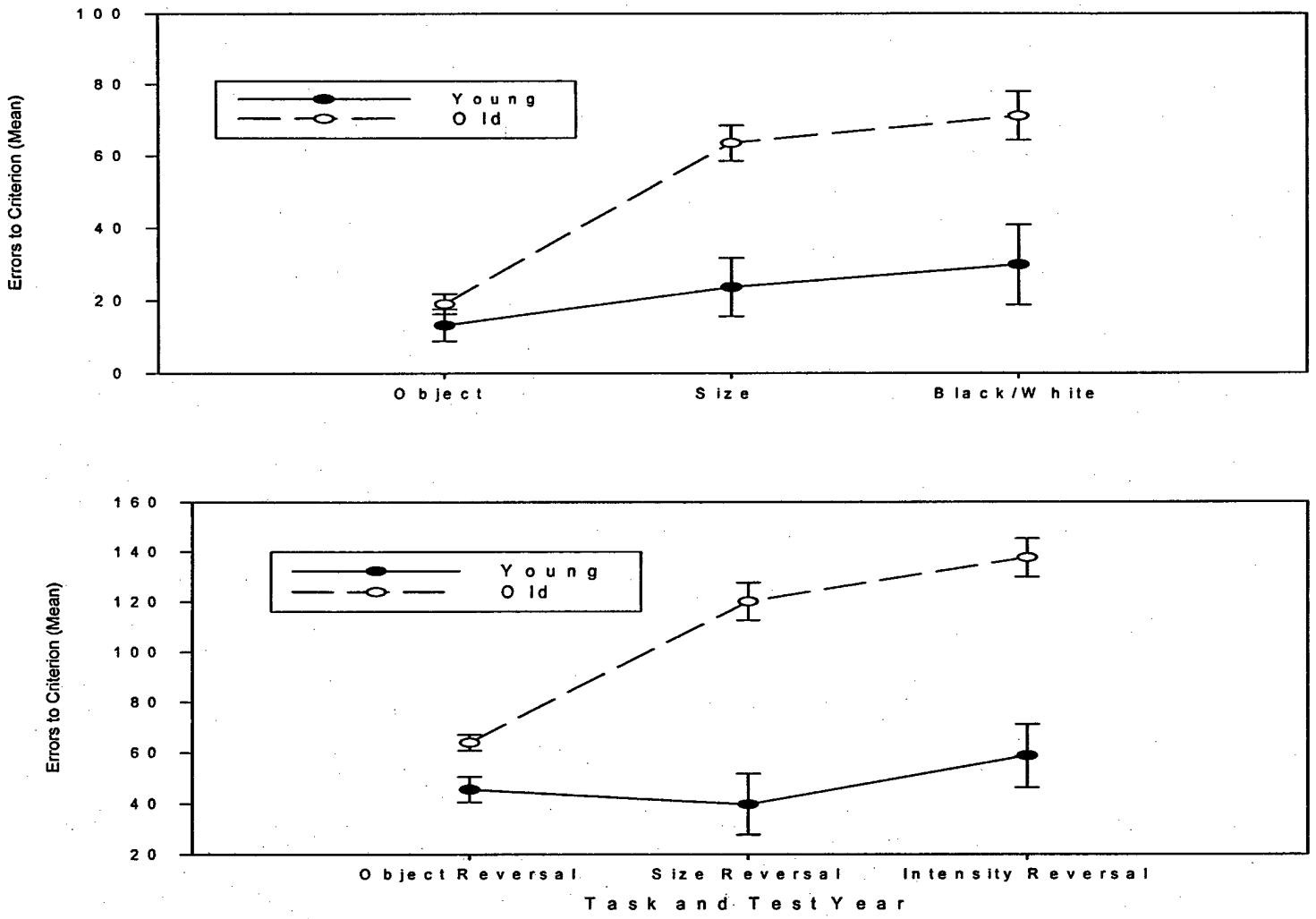


Figure 4 Age differences in discrimination learning (top) and reversal learning (bottom) over the course of three years. The figures were based only on the subjects that completed the entire study.

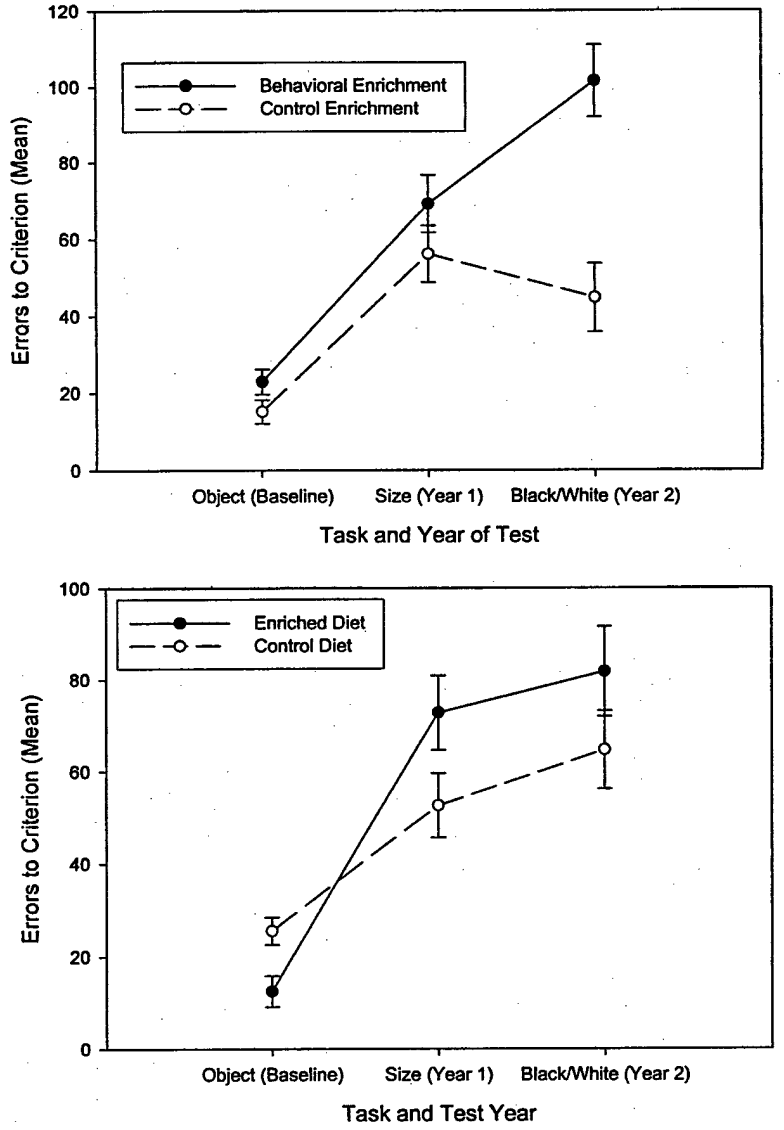


Figure 5 Longitudinal changes in reversal learning as a function of behavioral enrichment (top) and food (bottom).